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Europäisches Patentamt  
European Patent Office  
Office européen des brevets

11

Publication number:

**0 028 033**  
**A2**

12

## EUROPEAN PATENT APPLICATION

21

Application number: 80106685.3

22

Date of filing: 30.10.80

51

Int. Cl. 3: **C 12 N 15/00, C 12 P 21/02,**  
**C 12 N 1/20, C 07 H 21/04**  
**// C12R1/19**

30

Priority: 30.10.79 JP 139289/79  
19.03.80 JP 33931/80

71

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43

Date of publication of application: 06.05.81  
Bulletin 81/18

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84

Designated Contracting States: AT BE CH DE FR GB IT  
LI NL SE

74

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Novel DNA, cloned DNA, recombinant plasmid containing the DNA, microorganism containing the recombinant plasmid and process for their production.

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The invention relates to a DNA which codes for a polypeptide with interferon activity, a cloned DNA showing complementarity to human interferon mRNA, a recombinant plasmid containing such DNA, a microorganism containing the recombinant plasmid and a process for producing said DNA, said cloned DNA, said recombinant plasmid and said microorganism.

**EP 0 028 033 A2**

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15 Our Ref.: P 861 EP (Ra/fw) October 30, 1980  
Case: 265-Europ.

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"Novel DNA, Cloned DNA, Recombinant Plasmid Containing  
the DNA, Microorganism Containing the Recombinant Plasmid  
and Process for their Production"

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Priorities: October 30, 1980, Japan, No. 139 289/79  
March 19, 1980, Japan, No. 33 931/80

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The present invention relates to a DNA which codes for a  
polypeptide with interferon activity and to a recombinant  
plasmid containing the DNA. The present invention also  
pertains to a microorganism containing the recombinant  
35 plasmid.

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Interferon is a glycoprotein (molecular weight approx. 20,000) with antiviral activity, discovered by Isaacs and Lindenmann in 1957. Subsequent studies have indicated antitumor activity of the substance in addition to antiviral activity and hence a wide clinical application of this substance is expected. For instance, it has been reported that interferon may be effectively used to treat various viral diseases, osteosarcoma and mammary carcinoma.

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However, because of its high species-specificity, only the interferon derived from human cells can be used for human application. At present, the interferon which is being used for administration has a relative activity of about  $10^6$  (International units) per 1 mg, which corresponds to a purity of about 0.1 - 0.01%.

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Moreover, the use of the interferon is quite limited because of difficulties in mass production. At present only about 1% of the interferon requirement even for clinical tests ( $10^{13}$  units per annum) can be met. For these reasons there is a great need to develop technology to produce human interferon in high purity, with ease and in large quantities.

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To this end, a novel technique has been developed for producing interferon with ease and in a large quantity by inserting a human interferon gene into a plasmid DNA (for instance plasmid DNA derived from Escherichia coli) with recombinant DNA (deoxyribonucleic acid) technology.

35

1 In accordance with the present invention, a DNA  
which codes for a polypeptide with interferon activity is  
prepared using the human interferon messenger RNA as a  
template and a novel recombinant plasmid containing the DNA  
5 is prepared. In addition, the recombinant plasmid may be  
inserted into a host microorganism.

The DNA which codes for a polypeptide with  
interferon activity and the recombinant plasmid containing  
the DNA have been obtained for the first time by the present  
10 inventors. The DNA and the recombinant plasmid may be used,  
inter alia, for amplification of human interferon in bacteria  
such as Escherichia coli. Such bacteria are then useful for  
the production of human interferon in large quantities at  
low cost.

15 The DNA and the recombinant plasmid of the present  
invention are prepared by the following general procedure.

First, cytoplasmic RNA is extracted from (1) human  
fibroblast, MG63 cells or others induced by poly(I): poly(C)

20 (a double stranded RNA composed of polyinosinic acid  
and polycytidylic acid) or other inducers, (2) human leucocyte,  
lymphoblastic cells, NAMALWA cells or others induced by  
Sendai virus or other inducers, or (3) lymphocytes induced  
by various mitogens or other inducers. From this RNA, the  
human interferon messenger RNA (hereinafter messenger RNA  
25 is referred to as mRNA) containing poly A (polyadenylic acid)  
is isolated. A double stranded DNA is synthesized, for  
example, by reverse transcriptase, with the mRNA preparation  
having high interferon mRNA activity as a template.

30 A recombinant is obtained by inserting the synthesized DNA  
into a vector DNA such as Escherichia coli plasmid DNA by  
the technique of in vitro DNA recombination. The recombinant  
is labelled with a radio isotope for use as a probe.

Recombinant plasmids having an inserted portion which is

complementary to the human interferon mRNA are selected. The DNA which codes for a polypeptide with interferon activity is recovered from the recombinant plasmid and the base sequence of the DNA is determined.

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Fig. 1 illustrates restriction endonuclease maps of:

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(a) a gene which shows complementarity to the human fibroblast interferon mRNA in the recombinant #319 used to make a novel recombinant plasmid #319-13; and

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(b) a gene which shows complementarity to the human fibroblast interferon mRNA in the novel recombinant plasmid #319-13.

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The present invention relates to a DNA which codes for a polypeptide with interferon activity, a recombinant plasmid containing the DNA and a microorganism containing the recombinant plasmid.

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The DNA of the present invention may be a cloned DNA showing complementarity to the human interferon mRNA, a cloned DNA which codes for a polypeptide with interferon activity or a cloned DNA which codes for human interferon polypeptide. Especially a DNA which encompasses the entire coding region of the human fibroblast interferon mRNA is a preferred example of the DNA of the present invention.

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The recombinant plasmid of the present invention is a recombinant plasmid wherein the DNA mentioned above is inserted in a vector DNA such as pBR322, pCR1, pMB9 or pSC1.

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The recombinant plasmids named #319 and #319-13 are preferred examples of a recombinant plasmid according to the invention.

The DNA and the recombinant plasmid are inserted in a host microorganism and the transformant can be used to

produce a substance having interferon activity.

As the host microorganism, Escherichia coli X1776 is preferably used.

5 An example of the processes of producing the DNA, the recombinant plasmid and the transformant of the present invention is as follows.

10 First, human fibroblasts may be obtained from fetus-derived foreskin, or the like. A small amount of interferon is then added to a culture fluid of human fibroblasts to prime the interferon synthesis by human fibroblasts, to which poly(I): poly(C) is added to induce the synthesis of interferon mRNA. Cycloheximide is added simultaneously to increase the level of interferon mRNA. At an appropriate time (about 4 hours) after the human fibro-  
15 blasts have been superinduced in the above manner, cells are collected and destroyed and the nuclei are removed. Cytoplasmic total RNA is extracted with phenol, or the like. The RNA can also be extracted by destroying the whole cells, extracting both DNA and RNA with, for example, phenol, and  
20 degrading and removing the DNA with DNAase.

Further, interferon mRNA can also be extracted from MG63 cells induced by poly(I): poly(C) or other inducers, human leucocyte or lymphoblastic cells induced by Sendai virus or other inducers, and lymphocytes induced by various  
25 mitogens or other inducers.

The thus extracted RNA is dissolved in a salt solution of NaCl or KCl at a high concentration such as 0.5M and put on a column of oligo (dT) cellulose to adsorb mRNA having poly(A) on the column. Elution is carried out with  
30 water, a salt solution at a low concentration such as 10 mM Tris-HCl buffer, or the like to isolate mRNA having poly(A).

The isolated mRNA is fractionated by sucrose density gradient centrifugation. Interferon mRNA activity in each fraction is checked by determining interferon activity  
35 (antiviral activity) of the protein which is synthesized in oocytes of African claw toad (Xenopus laevis) after micro-injecting a part of the mRNA in each fraction. The determination of interferon activity is carried out according to the

method described in Japan J. Microbiol. 18, 449-456, (1974).

Then, a DNA showing complementarity to the mRNA is synthesized in vitro by a reverse transcriptase, which is obtained from avian myeloblastosis virus, using, as the  
5 template, an mRNA having the highest interferon mRNA activity.

The synthesis is carried out as follows.

An mRNA is reacted at an appropriate temperature (e.g. 37°C) for an appropriate period (e.g. 60 min.) with oligo(dT),  $MgCl_2$  (e.g. 5 mM), NaCl (e.g. 30 mM), mercapto-  
10 ethanol (e.g. 5 mM) and Tris-HCl buffer (e.g. pH 8.0, 40 mM) using a reverse transcriptase together with deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP) (e.g. 0.5 mM each) as substrates.

15 The thus obtained reaction product is subjected to deproteinization with, for example, phenol, and the template RNA is removed by alkali or ribonuclease treatment. A double stranded DNA is synthesized by a reverse transcriptase in a similar way as the synthesis of the DNA showing  
20 complementarity to mRNA described above except that mRNA is replaced by DNA and oligo(dT) is omitted.

By using Escherichia coli DNA polymerase I which can be obtained from Escherichia coli MRE 600, or the like, instead of reverse transcriptase, the same double stranded  
25 DNA can be synthesized.

After the double stranded DNA which is synthesized by the above described procedure is treated with Nuclease  $S_1$  which can be obtained from Aspergillus oryzae in the presence of  $ZnCl_2$  (e.g. 1 mM), sodium acetate buffer (e.g.  
30 0.1 M, pH 4.5), NaCl (e.g. 0.2 M), etc., deoxyadenine chains are formed at both 3' ends of the synthesized DNA by incubating with a terminal transferase purified from calf thymus in the presence of potassium cacodylate buffer (e.g. pH 7.6, 0.14 M), Tris (base) (e.g. 0.03 M), dithiothreitol (e.g.  
35 0.1 mM),  $CoCl_2$  (e.g. 1 mM) and dATP (e.g. 1 mM) at an appropriate temperature (e.g. 37°C) for an appropriate period (e.g. 20 min.)

On the other hand, a plasmid DNA which is used as a vector DNA, e.g. Escherichia coli plasmid pBR322 DNA [Gene vol. 2, p. 95-113 (1977)], is cleaved at one site by treating with a restriction endonuclease EcoRI, which can be obtained, for example, from Escherichia coli RY13, in the presence of Tris HCl buffer (e.g. pH 7.5, 10 mM),  $MgCl_2$  (e.g. 6 mM), NaCl (e.g. 0.1 M), mercaptoethanol (e.g. 6 mM), or the like and then treated with phage  $\lambda$ -derived exonuclease, which can be obtained, for example, from Escherichia coli W3102 ( $\lambda$  cI851 x 13), in the presence of Na-glycine buffer (e.g. pH 9.5, 0.1 M),  $MgCl_2$  (e.g. 5 mM), or the like. Thereafter deoxythymidine chains are formed at both 3' ends in the same way as for the above-described synthesized double stranded DNA by using dTTP instead of dATP.

Synthetic double stranded DNA and plasmid DNA which are chain-elongated at both 3' ends as described above are incubated at an appropriate temperature for an appropriate period with Tris-HCl buffer (e.g. pH 7.5, 50 mM), NaCl (e.g. 0.1 M), EDTA (e.g. 5 mM), or the like and hybridized with hydrogen bonds formed by adenine and thymine. Then, a transformable Escherichia coli strain, e.g. Escherichia coli  $\chi$ 1776 (Molecular Cloning of Recombinant DNA, Scott, W. A. & Werner, R. edited, Academic Press p. 99-114, 1977) is transformed with the hybridized DNA by the method of Enea et al. (J. Mol. Biol. vol. 96, p. 495-509, 1975) or the like.

In the novel recombinant plasmid DNA thus obtained, there exists a vector DNA gene, e.g.  $\beta$ -lactamase (enzyme that destroys ampicillin) gene, of Escherichia coli plasmid pBR322. Therefore, the transformed Escherichia coli shows resistance to ampicillin. The following technique is used to pick up a strain with a novel recombinant having a gene which shows complementarity to the human interferon messenger RNA among these ampicillin resistant strains.

First, [ $^{32}P$ ] labelled DNA is synthesized with the RNA having interferon mRNA activity described above as a template and the DNA is hybridized with mRNA extracted, without induction by poly(I): poly(C) (therefore, interferon mRNA synthesis is not induced), from the human fibroblasts



by incubating at a high temperature (e.g. 65°C) in a reaction mixture containing, for example NaCl (e.g. 0.5 M). Then, the hybridized DNA (Probe A) and non-hybridized DNA (Probe B) are separated by hydroxyapatite column chromatography.

5 Next, filter-fixed DNAs of transformants are hybridized separately with Probe B or Probe A according to the technique of Grunstein-Hogness (Proc. Nat. Acad. Sci. USA, vol. 72, p. 3961-3965, 1975) and strains having a DNA hybridizable with Probe B but not or barely with Probe A are discerned  
10 by autoradiography.

Then, plasmid DNA is isolated from each of the discriminated strains and hybridized with mRNA having interferon mRNA activity by incubating at a high temperature (e.g. 53°C) in the presence of 80% (w/v) formamide, 0.4 M  
15 NaCl, etc. Since the mRNA hybridized with cDNA portion of the plasmid DNA from the above-described strain can be retained on a nitrocellulose filter, whereas unhybridized mRNA can not under certain conditions (refer to Example below and Nygaard, A.P. & Hall, B. D., Biochem. Biophys.  
20 Res. Commun. Vol. 12, p. 98-104, 1963) this mRNA can be recovered selectively from the filter at a high temperature (e.g. 60°C) in a solution such as 90% (v/v) formamide and thereafter injected into oocytes of Xenopus laevis.

When interferon is synthesized in the oocytes,  
25 the DNA used for hybridization must contain a DNA which is complementary to interferon mRNA; and by this method, a recombinant plasmid DNA having a gene showing complementarity to the human fibroblast interferon mRNA can be isolated.

The recombinant plasmid DNA obtained above or  
30 segments cleaved with a restriction endonuclease are labelled with a radio isotope such as <sup>32</sup>P by the Nick translation method (Rigby, et al., J. Mol. Biol. vol. 113, p. 237-251, 1977), or the like, and used as a probe to obtain Escherichia coli strains containing a recombinant plasmid having the interferon mRNA sequence from the above ampicillin resistant  
35 strains in the same way as described above. Several strains thus obtained are cultured and the plasmid DNA is isolated therefrom. The plasmid DNA is cleaved with a restriction

endonuclease to obtain the inserted DNA. The length of the inserted DNA is investigated to obtain a plasmid having an inserted DNA coding the entire region of the interferon protein. Primary structure of the inserted DNA of one of recombinant plasmids isolated by the above method is determined according to the Maxam-Gilbert method (Proc. Nat. Acad. Sci. U.S.A. vol. 74, p. 560-564, 1977) and is illustrated in the following Example. It has thus been shown that the recombinant plasmid of the invention contains the entire coding region of the human fibroblast interferon mRNA.

As outlined above, a DNA which codes for human fibroblast interferon polypeptide, especially a DNA which encompasses the entire coding region of the human fibroblast interferon mRNA, a recombinant plasmid containing the DNA and a microorganism containing the plasmid are prepared.

The base sequence of the DNA obtained above and the corresponding peptide sequence are illustrated in Table 5 below.

The base sequence in Table 5 is a preferred example for the expression of the DNA which codes for human interferon polypeptide. Since the amino acids in the peptide sequence in Table 5 may be coded for by a base triplet other than those in Table 5, base sequences of the DNA which codes for human interferon polypeptide other than that in Table 5 are also included in the present invention.

The determination of the base sequence of the DNA which codes for human interferon polypeptide according to the present invention has enabled the chemical synthesis of such DNA.

The present novel recombinant plasmids having a gene which encompasses at least the entire coding region of the human fibroblast interferon mRNA are very useful because they enable mass production of interferon in Escherichia coli or in eukaryotic cells which can be grown on a large scale.

Recombinant plasmids containing a DNA using, as a template, leucocyte mRNA or immune interferon mRNA can be prepared by the same method as mentioned above and such

plasmids are also expected to be useful for the mass production of interferon.

One specific embodiment of the present invention is illustrated by the following representative example.

5

Example

After priming of human fibroblasts by overnight incubation with MEM culture medium (product of Nissui Seiyaku Co., Ltd., Japan) containing human interferon which is prepared according to the method described in Proc. Nat. Acad. Sci. USA, 73, 520-523 (1976) (25 U/ml), the fibroblasts were superinduced by adding 10 µg/ml of poly(I): poly(C) (product of Galbiochem Co., USA) and 5 µg/ml of cycloheximide to the medium. The priming and superinduction are carried out according to the methods described in Brit. J. Exp. Path., 39, 452-458 (1958) and Antimicrob. Agents Chemother., 2, 476-484 (1972), respectively.

After 4 hours,  $1.5 \times 10^9$  superinduced human fibroblasts were destroyed by Teflon homogenizer (sold by Takashima Shoten Co., Japan) at a temperature of 0 to 4°C in the presence of 0.3% NP-40 (product of Daiichi Kagaku Co., Japan) and 50 µg/ml heparin in RSB buffer (10 mM Tris-HCl, pH 7.5; 10 mM NaCl; 1.5 mM MgCl<sub>2</sub>). Nuclei were removed by centrifugation at 3000 rpm and 4°C for 10 minutes and 9.6 mg of cytoplasmic RNA was obtained by extraction 3 times with phenol.

The cytoplasmic RNA was precipitated with 67% ethanol in the presence of 0.1M NaCl, dissolved in 10 ml of 1 mM EDTA solution and incubated at 65°C for 2 minutes. Then, 2.5 ml of a salt solution at a high concentration (0.5 M Tris-HCl, pH 7.5; 1 M NaCl; 50 mM EDTA) was added to the above solution and the mixture was put on a column packed with 0.15 g of an oligo(dT) cellulose (product of P-L Biochemicals Co., USA) to adsorb mRNA containing poly(A). Elution was then carried out with a salt solution at a low concentration (10 mM Tris-HCl, pH 7.5) and water to isolate 250 µg of mRNA containing poly(A).

The mRNA was precipitated with 67% ethanol in the presence of 0.1M NaCl and dissolved in 0.5 ml of 1 mM EDTA solution. The solution was incubated at 65°C for 2 minutes, subjected to centrifugation through a 5 - 25% sucrose-density gradient containing 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl and 1 mM EDTA (rotated at 35,000 rpm using the SW40 rotor of Beckmann Co., U.S.A.) at 4°C for 16 hrs. and fractionated into 20 fractions.

The interferon mRNA activity of each of these fractions was determined as mentioned above, and the results are shown in Table 1 below.

Table 1

15	Fraction No.	Interferon Activity
	9	< 50 units/ml
	10	44
	11	550
20	12	52

The mRNA in Fraction No. 11 was approximately 5 µg. This mRNA and a reverse transcriptase were incubated at 37°C for an hour in 20 µl of a reaction mixture consisting of 5 µg mRNA; 0.5 mM dATP; 0.5 mM dTTP; 0.5 mM dGTP; 0.5 mM dCTP; 1 µg oligo(dT) (product of P-L Biochemicals Co., USA); 8 units reverse transcriptase (derived from Avian myeloblastosis virus, for example, product of Life Science Inc. Florida, USA); 5 mM MgCl<sub>2</sub>; 30 mM NaCl; 5 mM mercaptoethanol; and 40 mM Tris-HCl (pH 8.0) and then deproteinized with phenol. After RNA was removed by treatment with 0.3 N NaOH at 37°C for 15 hours, the synthesized single stranded DNA was incubated at 37°C in 20 µl of a reaction mixture [the same mixture as described above except that mRNA and oligo(dT) were omitted] for one hour to synthesize 1.5 µg of a double stranded DNA.

The double stranded DNA was treated with Nuclease S<sub>1</sub> (product of Bethesda Research Laboratories Inc., USA which is referred to as BRL, hereinafter) in 50 µl of a reaction mixture (1.5 µg double stranded DNA; 1 mM ZnCl<sub>2</sub>; 0.1 M sodium acetate, pH 4.5; 0.2 M NaCl; 0.05 unit S<sub>1</sub>) at 37°C for 30 minutes and the enzyme was removed by phenol extraction. The DNA was precipitated with ethanol and then treated with a terminal transferase in 20 µl of a reaction mixture consisting of 1.5 µg DNA; 0.14 M potassium cacodylate, pH 7.6; 0.03 M Tris (base); 0.1 mM dithiothreitol; 1 mM CoCl<sub>2</sub>; 1 mM dATP; and 1 unit terminal transferase (product of BRL) at 37°C for 20 minutes to obtain about 1.5 µg of a product wherein 100 deoxyadenosine chains were formed at both 3' ends of the double-stranded DNA.

In an alternative procedure, 10 µg of Escherichia coli plasmid pBR322 DNA (product of BRL) was treated at 37°C for 2 hours with a restriction endonuclease EcoRI in 100 µl of a reaction mixture consisting of 10 mM Tris-HCl, pH 7.5; 6 mM MgCl<sub>2</sub>; 0.1 M NaCl; 6 mM mercaptoethanol; and 10 units EcoRI (product of BRL) leading to cleavage at the only

cutting site in pBR322 DNA. The cut plasmid DNA was treated with an exonuclease derived from phage λ in 200 µl of a reaction mixture consisting of 10 µg DNA; 0.1 M Na-glycine, pH 9.5; 5 mM MgCl<sub>2</sub>; 50 µg/ml albumin (product of Merck & Co., USA); and 17.5 units λ exonuclease (product of Miles Laboratories Inc., USA) at 0°C for 90 minutes and the enzyme was removed by phenol extraction. The DNA was treated with a terminal transferase in 50 µl of a reaction mixture [10 µg DNA; 0.14 M potassium cacodylate, pH 7.6; 0.03 M Tris (base); 0.1 mM dithiothreitol; 1 mM CoCl<sub>2</sub>; 1 mM dTTP; 2 units terminal transferase] at 37°C for 20 minutes to obtain about 0.5 µg of a product wherein 100 deoxythymidine chains were formed at both 3' ends of plasmid pBR322 DNA described above.

Then, 0.02 µg of the synthesized double stranded DNA obtained above, and 0.1 µg of the plasmid pBR322 DNA were incubated for hybridization in a solution containing 0.1 M NaCl, 50 mM Tris-HCl (pH 7.5) and 5 mM EDTA at 65°C

for 2 minutes, at 45°C for one hour, at 37°C for one hour and at room temperature for one hour. Then, Escherichia coli X1776 was transformed with the hybridized recombinant following the method of Enea et al.

5 About 4,000 ampicillin-resistant strains were isolated by this method. 3,600 resistant strains were chosen, and the DNA of each strain was fixed on nitrocellulose filters in duplicate (Grunstein-Hogness Method).

10 In a further procedure, [ $^{32}\text{P}$ ] labelled single stranded DNA was synthesized (about 0.44  $\mu\text{g}$ , specific radioactivity approx.  $6 \times 10^8$  c.p.m./ $\mu\text{g}$ ) by reverse transcriptase in the same way as that for single stranded DNA mentioned above (dCTP was labelled with  $^{32}\text{P}$ ) using the interferon mRNA fraction (about 10  $\mu\text{g}$ ) which had been extracted and partially  
15 purified as described above, as a template. The DNA was hybridized in 50  $\mu\text{l}$  of a reaction mixture (25  $\mu\text{g}$  mRNA; 0.45  $\mu\text{g}$  single stranded DNA labelled with  $^{32}\text{P}$ ; 0.5 M NaCl; 25 mM Pipes buffer, pH 6.5) at 65°C for 40 hours with 25  $\mu\text{g}$  of mRNA extracted from human fibroblasts which had not been  
20 induced by poly(I): poly(C). The latter mRNA was prepared by the same method used to extract poly(I): poly(C)-induced mRNA. The reaction mixture was put on a column packed with 0.2 g of a hydroxyapatite, and elution was first carried out with 5 ml of 0.14 M phosphate buffer (pH 6.5) to elute the  
25 single stranded DNA, and then with 5 ml of 0.4 M phosphate buffer to elute the DNA hybridized with RNA. As a result, the DNA (about 90% of the total) (Probe A) which hybridized with the mRNA mentioned above, and the DNA (about 10% of the total) (Probe B) which did not hybridize were isolated.

30 Each probe was then hybridized separately with the above DNA fixed on the nitrocellulose filters according to the Grunstein-Hogness method. Four strains were identified which reacted mainly to Probe B but little to Probe A under autoradiography.

35 Table 2 shows the extent of reaction of the DNAs from the four strains to each probe as revealed by autoradiogram.

Table 2

Ampicillin-resistant strains	Extent of Reaction of Probe with DNA in the strains	
	Probe A	Probe B

# 319	+	+	+	+	+
# 644	+				
# 746	-				
#3578	+				

Plasmid DNA was isolated from cells of the four strains by the method of Currier and Nester (Analyt. Biochem. vol. 76, p. 431-441, 1976). Then, these DNAs were hybridized with the interferon mRNA as follows.

First, 5 µg of plasmid DNA was linearized by incubating with restriction endonuclease Hind III, which can be obtained from Haemophilus influenzae Rd, in 50 µl of a reaction mixture consisting of 10 mM Tris-HCl, pH 7.5; 6 mM MgCl<sub>2</sub>; 50 mM NaCl; 6 mM mercaptoethanol; and 5 units Hind III (product of BRL) at 37°C for 2 hours. After deproteinization by phenol extraction, the DNA was precipitated with ethanol and dissolved in 20 µl of 80% (w/v) formamide. The solution was denatured at 85°C for 10 minutes and was then incubated in a solution consisting of 2.5 µg mRNA, 20 µl 80% (w/v) formamide, 20 mM Pipes buffer (pH 6.5), 0.4 M NaCl and 5 mM EDTA, at 53°C. Four hours later the mixture was mixed with 0.4 ml of 3 x SSC (1 x SSC corresponds to 0.15 M NaCl, 0.015 M sodium citrate) at 0°C, and was filtered through a nitrocellulose filter (diameter : 1 cm, pore size : 0.45 µm) at a rate of about 0.5 ml per minute. After washing the filter with about 1.5 ml of 2 x SSC, the filter was immersed in a solution consisting of 0.6 ml of 90% (v/v) formamide, 20 mM Pipes buffer, 0.1% SDS (sodium dodecylsulfate) and 5 mM EDTA. Incubation of the filter at 60°C for 2 minutes and the removal of the solution were repeated 3 times and the RNA eluted from the nitrocellulose filter into the solution (1.8 ml) was precipitated with ethanol in the presence of

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0.1 M NaCl. The mRNA containing poly(A) was isolated from the RNA by using oligo(dT) cellulose column chromatography, dissolved in a mixture of 3  $\mu$ l of 10 mM Tris-HCl (pH 7.5) and 88 mM NaCl and injected into the oocytes of Xenopus laevis. After 15 hours the interferon synthesized in the oocytes was determined (antiviral activity).

Table 3 shows the interferon mRNA activity of the mRNA which has hybridized with the DNA derived from the four bacterial strains mentioned above.

Table 3

Bacterial strain	Interferon mRNA activity (unit/ml)
# 319	360
# 644	< 10
# 746	15
#3578	< 10
pBR322DNA	< 10

Five  $\mu$ g of plasmid DNA obtained from strain #319 by the Currier and Nester method was cleaved with restriction endonuclease Hind III in the same manner as mentioned above.

The DNA and the recombinant plasmid  $\beta$ GpBR322 DNA (the vector was pBR322) (obtained from the Institute for Molecular Biology I of University of Zurich or prepared by the method described in Nature 281, 40-46, 1979) containing rabbit  $\beta$ -globin gene, separately or as a mixture, were hybridized with a mixture of rabbit globin mRNA (obtained from rabbit red blood cells) (1  $\mu$ g) and interferon mRNA (2.5  $\mu$ g) obtained from human fibroblasts under the same conditions as mentioned above. The mRNA which formed a hybrid was injected into the oocytes of Xenopus laevis. The oocytes were then incubated for 15 hours in Barth's culture medium (J. Embryol. Exp. Morph. 7, 210, 1959) containing [ $^3$ H] labelled histidine, and [ $^3$ H] labelled globin was isolated by acrylamide gel electrophoresis and determined quantitatively by fluorography



according to the method described in Eur. J. Biochem. 46, 83-88, (1974). The interferon was determined by antiviral activity as described above. The synthesis of rabbit  $\beta$ -globin and the human interferon was determined in this way.

5 The result is shown in Table 4 below.

Table 4

10	D N A	Synthesized interferon activity	Amount of globin synthesized
	# 319	200 (units/ml)	-
	$\beta$ GpBR322	35	+ + + +
15	mixture of both plasmids	160	+ + +

From the results of this experiment it has been established that DNA of #319 has DNA (the interferon gene) which forms a hybrid specifically with the interferon mRNA.

20 The DNA of #319 was cleaved with several restriction endonucleases and a restriction endonuclease map, Fig. 1(a), was made by agarose electrophoresis.

Restriction endonucleases, Pst I, Bgl II and Hind III (sold by BRL, etc.) cleave #319 DNA at the sites illustrated in Fig. 1 (a).

25 The segments obtained by cleaving #319 DNA with restriction endonucleases Pst I and Bgl II were isolated and purified by gel electrophoresis according to the method of Tabak & Flavell (Nucleic Acids Research, vol. 5, p. 2321-2332, 1978). The segments were labelled with  $^{32}$ P according to the method of Rigby, et al. (J. Mol. Biol. vol. 113, p. 237-251, 1977) and the labelled segment was used as a probe. Several strains containing a plasmid which shows complementarity to the probe were isolated from the above  
30 ampicillin-resistant strains according to the above method of Grunstein & Hogness (Proc. Nat. Acad. Sci. U.S.A., vol. 72, p. 3961-3965, 1975), namely the colony hybridization method. Plasmid DNAs were obtained from each of the strains according

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to the above method of Currier-Nester and the inserted portions thereof were cleaved with a restriction endonuclease such as Hind III. The cut plasmid DNA segments were compared in length and the longest plasmid DNA segment was selected.

5 The plasmid was named #319-13.

The restriction endonuclease map of the plasmid is illustrated in Fig. 1 (b), which substantiates that the novel plasmid has an mRNA sequence containing the mRNA sequence of #319. Primary structure (base sequence) of the mRNA sequence inserted in the plasmid of #319-13 was determined by the method of Maxam-Gilbert (Proc. Nat. Acad. Sci, U.S.A. vol. 74, p. 560-564, 1977). The primary structure is given in Table 5 below.

10

Table 5

	-20		-10		1
	MET THR ASN LYS CYS LEU LEU GLN ILE ALA LEU LEU LEU CYS PHE SER THR THR ALA LEU SER MET SER TYR				
5	GTG AAC ATG ACC AAC AAG TGT CTC CTC CAA ATT GCT CTC CTG TTG TGC TTC TCC ACT ACA GCT CTT TCC ATG AGC TAC				
	CAG TTG TAC TGG TTG TTC ACA GAG GAG GTT TAA CGA GAG GAC AAC ACG AAG AGG TGA TGT CGA GAA AGG TAC TCG ATG				
	20	40	60		
	ASN LEU LEU GLY PHE LEU GLN ARG SER SER ASN PHE GLN CYS GLU LYS LEU LEU TRP GLN LEU ASN GLY ARG LEU GLU				
	AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT CAG TGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA				
	TTG AAC GAA CCT AAG GAT GTT TCT TCG TCG TTA AAA GTC ACA GTC TTC GAG GAC ACC GTT AAC TTA CCC TCC GAA CTT				
	80	100	120	140	
10	LYR CYS LEU LYS ASP ARG MET ASN PHE ASP ILE PRO GLU GLU ILE LYS GLN LEU GLN GLN PHE GLN LYS GLU ASP ALA				
	TAT TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC				
	ATA ACG GAG TTC CTG TCC TAC TTG AAA CTG TAG GGA CTC CTC TAA TTC GTC GAC GTC GTC AAG GTC TTC CTC CTG CGG				
	160	180	200	220	
	ALA LEU THR ILE TYR GLU MET LEU GLN ASN ILE PHE ALA ILE PHE ARG GLU ASP SER SER SER THR GLY TRP ASN GLU				
	GCA TTG ACC ATC TAT GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT AGC ACT GGC TGG AAT GAG				
15	CGT AAC TGG TAG ATA CTC TAC GAG GTC TTG TAG AAA CGA TAA AAG TCT GTT CTA AGT AGA TCG TGA CCG ACC TTA CTC				
	240	260	280	300	
	THR ILE VAL GLU ASN LEU LEU ALA ASN VAL TYR HIS GLN ILE ASN HIS LEU LYS THR VAL LEU GLU GLU LYS LEU GLU				
	ACT ATT GTT GAG AAC CTC CTG GCT AAT GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA GTC CTG GAA GAA AAA CTG GAG				
	TGA TAA CAA CTC TTG GAG GAC CGA TTA CAG ATA GTA GTC TAT TTG GTA GAC TTC TGT CAG GAC CTT CTT TTT GAC CTC				
	320	340	360	380	
	LYS GLU ASP PHE THR ARG GLY LYS LEU MET SER SER LEU HIS LEU LYS ARG TYR TYR GLY ARG ILE LEU HIS TYR LEU				
20	AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG CAC CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG				
	TTT CTT CTA AAG TGG TCC CCT TTT GAG TAC TCG TCA GAC GTG GAC TTT TCT ATA ATA CCC TCC TAA GAC GTA ATG GAC				
	400	420	440	460	
	LYS ALA LYS GLU TYR SER HIS CYS ALA TRP THR ILE VAL ARG VAL GLU ILE LEU ARG ASN PHE TYR PHE ILE ASN ARG				
	AAG GCC AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC CTA AGG AAC TTT TAC TTC ATT AAC AGA				
25	TTG CGG TTC CTC ATG TCA GTG ACA CGG ACC TGG TAT CAG TCT CAC CTT TAG GAT TCC TTG AAA ATG AAG TAA TTG TCT				
	480	500	520	540	
	LEU THR GLY TYR LEU ARG ASN				
	CTT ACA GGT TAC CTC CGA AAC TGA AGA TCT CCT AGC CTG TGC CTC TGG GAC TGG ACA ATT GCT TCA AGC ATT CTT CAA				
	GAA TGT CCA ATG GAG GCT TTG ACT TCT AGA GGA TCG GAC ACG GAG ACC CTG ACC TGT TAA CGA AGT TCG TAA GAA GTT				
	560	580	600	620	
	CCA GCA GAT GCT GTT TAA GTG ACT GAT GGC TAA TGT ACT GCA TAT GAA AGG ACA CTA GAA GAT TTT GAA ATT TTT ATT				
30	GGT CGT CTA CGA CAA ATT CAC TGA CTA CCG ATT ACA TGA CGT ATA CTT TCC AGT GAT CTT CTA AAA CTT TAA AAA TAA				
	640	660	680	700	
	AAA TTA TGA GTT ATT TTT ATT TAT TTA AAT TTT ATT TTG GAA AAT AAA TTA TTT TTG GTG CAA AAG TCA AAA AAA				
	TTT AAT ACT CAA TAA AAA TAA ATA AAT TTA AAA TAA AAC CTT TTA TTT AAT AAA AAC CAC GTT TTC AGT TTT TTT				
	720	740	760		

35

The DNA sequence permits prediction of the entire amino acid sequence for human fibroblast interferon (amino acids 1-166) and its putative signal peptide (amino acids -21 to -1) as shown in the line above the DNA sequences.

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It is important that in the sequence there exist without any errors the base sequence (three base pairs) corresponding to the amino acid sequence from the amino-terminal to 13th amino acid of the human fibroblast interferon reported by Knight, et al. (Science vol. 207, p. 525-526, 1980). This fact establishes that the #319-13 plasmid of the present invention has the human fibroblast interferon mRNA sequence.

Further, it is apparent from the data of the primary sequence that the plasmid encompasses the entire coding region of the protein of the above mRNA and probably the coding region of the signal peptide.

Therefore, transformation of the plasmid or mRNA inserted therein to other expression plasmids enables a host such as Escherichia coli to produce interferon. For such purposes, the #319-13 plasmid which is named TpIF 319-13, transformed in Escherichia coli X1776, has been deposited with the American Type Culture Collection, Rockville, Maryland, U.S.A. under accession number ATCC 31712 and is freely available to the public.

What is Claimed is

1. A DNA which codes for a polypeptide with interferon activity.
2. A cloned DNA showing complementarity to human interferon messenger RNA.
3. The cloned DNA according to claim 2, wherein the messenger RNA is human fibroblast interferon messenger RNA.
4. A cloned DNA which codes for human interferon polypeptide.
5. The cloned DNA according to claim 4, wherein the polypeptide is human fibroblast interferon polypeptide.
6. A recombinant plasmid wherein a DNA showing complementarity to human interferon messenger RNA is inserted in a vector DNA.
7. The recombinant plasmid according to claim 6, wherein the messenger RNA is human fibroblast interferon messenger RNA.
8. The recombinant plasmid according to claims 6 or 7, wherein the plasmid is an Escherichia coli plasmid.
9. The recombinant plasmid according to claim 8, wherein the plasmid is selected from pBR322, pCRL, pMB9 and pSC1.
10. The recombinant plasmid TpIF 319-13.
11. A microorganism containing the recombinant plasmid defined in claim 6.
12. A microorganism containing the recombinant plasmid defined in claim 7.
13. The microorganism according to claim 11 which is Escherichia coli X1776.

14. The microorganism according to claim 12 which is Escherichia coli  $\chi$ 1776.

15. Escherichia coli  $\chi$ 1776/TpIF 319-13 ATCC 31712.

16. A process for producing a DNA which codes for a polypeptide with interferon activity by recombinant DNA technology.

17. The process according to claim 16, wherein the polypeptide is the human fibroblast interferon polypeptide.

18. A process for producing a DNA which codes for a polypeptide with interferon activity by using human interferon messenger RNA as a template.

19. The process according to claim 18, wherein the DNA is a cloned DNA showing complementarity to human interferon messenger RNA.

20. The process according to claim 19, wherein the messenger RNA is human fibroblast interferon messenger RNA.

21. A process for producing a recombinant plasmid, which comprises inserting a DNA showing complementarity to human interferon messenger RNA in a vector DNA.

22. The process according to claim 21, wherein the messenger RNA is human fibroblast interferon messenger RNA.

23. The process according to claim 21 or 22, wherein the vector DNA is an Escherichia coli plasmid.

24. The process according to claim 23, wherein the plasmid is selected from pBR322, pCR1, pMB9 and pSC1.

25. The process according to claim 21, wherein the recombinant plasmid is TpIF 319-13.

26. A process for producing a microorganism containing a recombinant plasmid defined in claim 21 or 22, which comprises transforming a microorganism with said

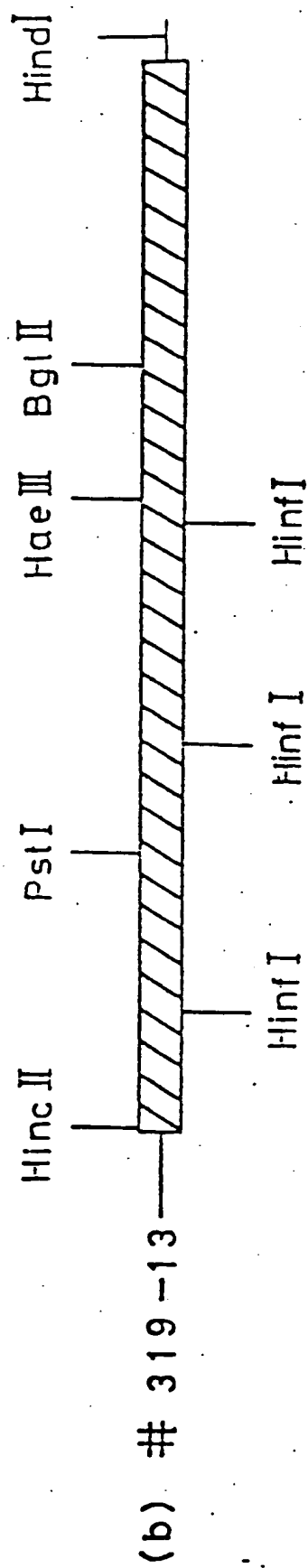
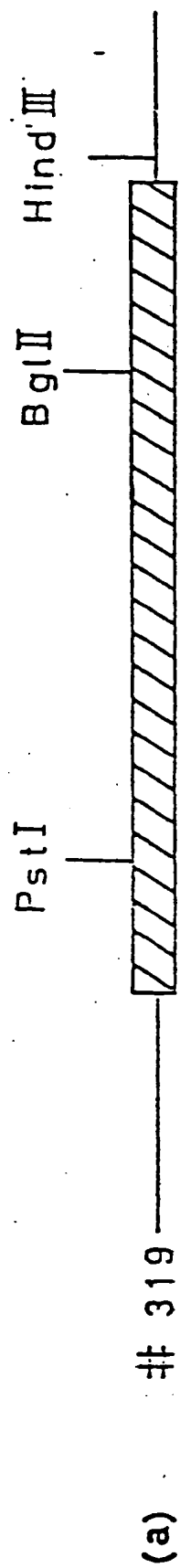
recombinant plasmid in a conventional manner.

27. The process according to claim 26, wherein the microorganism is Escherichia coli X1776.

28. The process according to claim 26, wherein the recombinant plasmid is TpIF 319-13.

29. The process according to claim 26, wherein the microorganism containing a recombinant plasmid is Escherichia coli X1776 TpIF 319-13 ATCC 31712.

Fig. 1



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(19)



Europäisches Patentamt  
European Patent Office  
Office européen des brevets

(11) Publication number:

0048 970  
A2

(12)

# EUROPEAN PATENT APPLICATION

(21) Application number: 81107621.5

(22) Date of filing: 24.09.81

(51) Int. Cl.<sup>3</sup>: C 12 N 15/00, C 12 P 21/02,  
C 12 N 1/20, C 07 H 21/04,  
C 07 C 103/52, A 61 K 45/02  
// C12R1/19, C12R1/125,  
C12R1/865

(30) Priority: 25.09.80 US 190799  
11.08.81 US 291892

(43) Date of publication of application: 07.04.82  
Bulletin 82/14

(54) Designated Contracting States: AT BE CH DE FR GB IT  
LI LU NL SE

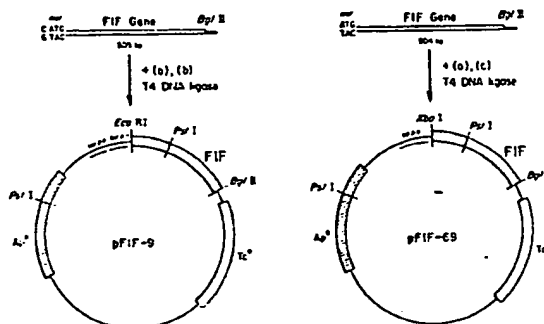
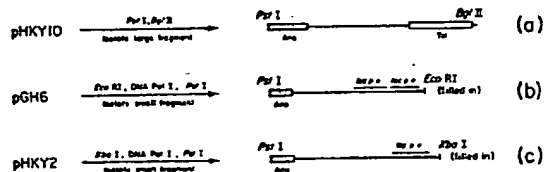
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(52) Polypeptides, process for their microbial production, intermediates therefor and compositions containing them.

(57) Microbially produced mature human fibroblast Interferon and means used in its production, i.e. recombinant DNA molecules coding for the amino acid sequences of fibroblast interferon, vectors capable of expressing fibroblast Interferon in microbial host organisms and host organisms transformed with these vectors.



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5 Polypeptides, process for their microbial production,  
intermediates therefor and compositions containing them

10 The present invention relates to the field of re-  
combinant DNA technology, i.e. to processes used in recom-  
binant DNA technology and to products obtained by these  
processes.

15 In a more detailed aspect the present invention re-  
lates to polypeptides, specifically to mature human fibro-  
blast interferon, to pharmaceutical compositions containing  
them and to a process for their preparation which comprises  
causing a culture of a microorganism transformed with a  
20 replicable microbial expression vehicle capable of expres-  
sing said polypeptides to grow up and express said poly-  
peptides. The present invention also comprises the expres-  
sion vehicles used in this process and the novel micro-  
organisms containing these expression vehicles as well as  
25 the processes for their preparation. Finally, the invention  
relates to DNA sequences comprising sequences coding for  
the amino acid sequence of a mature human fibroblast inter-  
feron.

30 Background of the invention

Human fibroblast interferon (FIF) is a protein which  
exhibits antiviral as well as a wide range of other bio-  
logical activities (for review see W.E. Stewart II, The  
35 Interferon System, Springer-Verlag, New York-Wien, 1979).

It has reportedly been purified to homogeneity as a single polypeptide with a molecular weight of 19000 - 20000 having a specific activity of  $2-10 \times 10^8$  units/mg (E. Knight, Proc. Natl. Acad. Sci. USA 73, 520-523 [1976];

5 W. Berthold et al., J. Biol. Chem. 253, 5206-5212 [1978]). The sequence of the 13 NH<sub>2</sub>-terminal amino acids of FIF has been determined to be Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu-Gln-Arg-Ser-Ser- (E. Knight et al., Science 207, 525-526 [1980]). Houghton et al. (Nucleic Acids Res. 8, 1913-

10 1931 [1980]) have used synthetic deoxyoligonucleotides (predicted from this amino acid sequence) to determine the sequence of the 276 5'-terminal nucleotides of FIF mRNA. Taniguchi et al. (Nature 285, 547-549 [1980]; Gene 10, 11-15 [1980]) and Derynck et al. (Nature 285, 542-547

15 [1980]) have recently been able to identify the nucleotide sequence of cloned cDNA copies of FIF mRNA in E. coli and have deduced therefrom the complete amino acid sequence of human FIF including a 21 amino acids signal sequence. The mature peptide is 166 amino acids long. Finally,

20 Taniguchi et al. (Proc. Natl. Acad. Sci. USA 77, 5230-5233 [1980]) have constructed a plasmid that directs expression in E. coli of the human FIF gene yielding mature FIF.

With the advent of recombinant DNA technology, the

25 controlled microbial production of an enormous variety of useful polypeptides has become possible. Already in hand are bacteria modified by this technology to permit the production of such polypeptide products such as somatostatin, the A and B chains of human insulin, human growth

30 hormone (Itakura et al., Science 198, 1056-1063 [1977]; Goeddel et al., Nature 281, 544-548 [1979]). More recently, recombinant DNA techniques have been used to occasion the bacterial production of proinsulin, thymosin  $\alpha_1$  and leukocyte interferon.

35

The workhorse of recombinant DNA technology is the plasmid, a non-chromosomal loop of double-stranded DNA found in bacteria and other microbes, oftentimes in multiple

copies per cell. Included in the information encoded in the plasmid DNA is that required to reproduce the plasmid in daughter cells (i.e., a "replicon") and ordinarily, one or more selection characteristics such as, in the case of  
5 bacteria, resistance to antibiotics which permit clones of the host cell containing the plasmid of interest to be recognized and preferentially grown in selective media. The utility of plasmids lies in the fact that they can be specifically cleaved by one or another restriction endo-  
10 nuclease or "restriction enzyme", each of which recognizes a different site on the plasmidic DNA. Thereafter heterologous genes or gene fragments may be inserted into the plasmid by endwise joining at the cleavage site or at reconstructed ends adjacent to the cleavage site. DNA  
15 recombination is performed outside the cell, but the resulting "recombinant" plasmid can be introduced into it by a process known as transformation and large quantities of the heterologous gene-containing recombinant plasmid are obtained by growing the transformant. Moreover, where the  
20 gene is properly inserted with reference to portions of the plasmid which govern the transcription and translation of the encoded DNA message, the resulting expression vehicle can be used to actually produce the polypeptide sequence for which the inserted gene codes, a process  
25 referred to as expression.

Expression is initiated in a region known as the promoter which is recognized by and bound by RNA polymerase. In some cases, as in the tryptophan or "trp" promoter preferred in the practice of the present invention, promoter  
30 regions are overlapped by "operator" regions to form a combined promoter-operator. Operators are DNA sequences which are recognized by so-called repressor proteins which serve to regulate the frequency of transcription initiation  
35 at a particular promoter. The polymerase travels along the DNA, transcribing the information contained in the coding strand from its 5' to 3' end into messenger RNA which is in turn translated into a polypeptide having the amino acid

sequence for which the DNA codes. Each amino acid is encoded by a nucleotide triplet or "codon" within what may for present purposes be referred to as the "structural gene", i.e. that part which encodes the amino acid sequence of the expressed product. After binding to the promoter, the RNA polymerase first transcribes nucleotides encoding a ribosome binding site, then a translation initiation or "start" signal (ordinarily ATG, which in the resulting messenger RNA becomes AUG), then the nucleotide codons within the structural gene itself. So-called stop codons are transcribed at the end of the structural gene whereafter the polymerase may form an additional sequence of messenger RNA which, because of the presence of the stop signal, will remain untranslated by the ribosomes. Ribosomes bind to the binding site provided on the messenger RNA, in bacteria ordinarily as the mRNA is being formed, and themselves produce the encoded polypeptide, beginning at the translation start signal and ending at the previously mentioned stop signal. The desired product is produced if the sequences encoding the ribosome binding site are positioned properly with respect to the AUG initiation codon and if all remaining codons follow the initiation codon in phase. The resulting product may be obtained by lysing the host cell and recovering the product by appropriate purification from other bacterial protein.

While isolation from donor fibroblasts has provided sufficient material for partial characterization and limited clinical studies with homogeneous leukocyte interferon, it is a totally inadequate source for the amounts of interferon needed for large scale clinical trials and for broad scale prophylactic and/or therapeutic use thereafter. Indeed, presently clinical investigations employing human fibroblast-derived interferons in antitumor and antiviral testing have principally been confined to crude (<1 percent pure) preparations of the material, and long lead times for the manufacture of sufficient quantities, even at unrealistic price levels, have critically delayed

investigation on an expanded front.

We perceived that application of recombinant DNA technology would be the most effective way of providing large quantities of fibroblast interferon which, despite the absence in material so produced of the glycosylation characteristic of human-derived material, could be employed clinically in the treatment of a wide range of viral and neoplastic diseases and have succeeded in producing mature human fibroblast interferon microbially, by constructing a gene therefor which could then be inserted in microbial expression vehicles and expressed under the control of microbial gene regulatory controls.

Our approach to obtaining a fibroblast gene involved the following tasks:

1. Partial amino acid sequences of human fibroblast interferon were used to construct sets of synthetic DNA probes the codons of which, in the aggregate, represented all the possible combinations capable of encoding the partial amino acid sequences.

2. Bacterial colony banks were prepared containing complementary DNA (cDNA) from induced messenger RNA. The probes of part (1) were used to prime the synthesis of radio-labelled single stranded cDNA for use as hybridization probes. The synthetic probes would hybridize with induced mRNA as template and be extended by reverse transcription to form induced, radio-labelled cDNA. Clones from the colony bank that hybridized to radio-labelled cDNA obtained in this manner have been investigated further to confirm the presence of a full-length interferon encoding gene. Any partial length putative gene fragment obtained was itself used as a probe for the full-length gene.

3. The full-length gene obtained above was tailored, using synthetic DNA, to eliminate any leader sequence that might prevent microbial expression of the mature polypeptide and to permit appropriate positioning in an expression vehicle relative to start signals and the ribosome binding site of a microbial promoter. Expressed interferon was purified to a point permitting confirmation of its character and determination of its activity.

10 In applying methods of recombinant DNA technology as outlined above a series of replicable plasmidic expression vehicles have been constructed which direct the high level synthesis in transformant microorganisms of a mature polypeptide with the properties of authentic human fibroblast  
15 interferon. The product polypeptide exhibits the amino acid sequence of such interferon and is active in in vitro testing despite the lack of glycosylation characteristic of the human-derived material. Reference herein to the "expression of mature fibroblast interferon" connotes the  
20 bacterial or other microbial production of an interferon molecule containing no glycosyl groups or a presequence that immediately attends mRNA translation of the human fibroblast interferon genome. Mature fibroblast interferon, according to the present invention, is immediately  
25 expressed from a translation start signal (ATG) which also encodes the first amino acid codon of the natural product. The presence or absence of the methionine first amino acid in the microbially expressed product is governed by a kinetic phenomenon dependent on fermentation growth conditions and/or levels of expression in the transformant host.  
30 Mature fibroblast interferon could be expressed together with a conjugated protein other than the conventional leader, the conjugate being specifically cleavable in an intra- or extracellular environment (see British Patent  
35 Publication No. 2007676A). Finally, the mature interferon could be produced in conjunction with a microbial "signal" peptide which transports the conjugate to the cell wall, where the signal is processed away and the mature poly-

peptide secreted.

Figures 1 to 5 appended hereto are described in the detailed text infra. Figure 6 schematically depicts the construction of plasmids coding for the direct expression of mature fibroblast interferon. Restriction sites and residues are as shown ("Pst I", etc.). "Ap<sup>R</sup>" and "Tc<sup>R</sup>" connote portions of the plasmid which express, respectively, ampicillin and tetracycline resistance. The legend "p o" is an abbreviation for "promoter operator".

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

##### A. Microorganisms employed

15

The work described involved use of the microorganism E. coli K-12 strain 294 (end A, thi<sup>-</sup>, hsr<sup>-</sup>, hsm<sub>k</sub><sup>+</sup>), as described in British Patent Publication No. 2055382 A. This strain has been deposited with the American Type Culture Collection, ATCC Accession No. 31446/ on Oct. 28, 1978. All recombinant DNA work was performed in compliance with applicable guidelines of the National Institutes of Health.

The invention although described in its most preferred embodiments with reference to E. coli K-12 strain 294, defined above, comprises also other known E. coli strains such as E. coli B, E. coli x 1776 and E. coli W 3110, or other microbial strains many of which are deposited and (potentially) available from recognized microorganism depository institutions, such as the American Type Culture Collection (ATCC). See also German Offenlegungsschrift 2644432. These other microorganisms include, for example, Bacilli such as Bacillus subtilis and other enterobacteriaceae among which can be mentioned as examples Salmonella typhimurium and Serratia marcescens, utilizing plasmids that can replicate and express heterologous gene sequences therein. Yeast, such as Saccharomyces cerevisiae, may also be employed to advantage as host organism in the



preparation of the interferon protein hereof by expression of genes coding therefor under the control of a yeast promoter.

5 B. General methods

Restriction enzymes were purchased from New England Biolabs and used as directed. Plasmid DNA was prepared by a standard cleared lysate procedure (D.B. Clewell, J. Bacteriol. 110, 667-676 [1972]) and purified by column chromatography on Biogel A-50M. DNA sequencing was performed using the method of Maxam and Gilbert (Methods Enzymol. 65, 499-560 [1980]). DNA restriction fragments were isolated from polyacrylamide gels by electroelution. DNA fragments were radiolabeled for use as hybridization probes by the random calf thymus DNA priming procedure of Taylor et al. (Biochim. Biophys. Acta 442, 324-330 [1976]). In situ colony hybridizations were performed by the Grunstein-Hogness procedure (Proc. Natl. Acad. Sci. USA 72, 3961-3965 [1975]).

C. Chemical synthesis of deoxyoligonucleotides

The deoxyoligonucleotides were synthesized by the modified phosphotriester method in solution (Crea et al., Proc. Natl. Acad. Sci. USA 75, 5765-5769 [1978]), using trideoxynucleotides as building blocks (Hirose et al., Tetrahedron Letters 28, 2449-2452 [1978]). The materials and general procedures were similar to those described by Crea et al., Nucleic Acids Res. 8, 2331-2348 [1980]. The six pools of primers (Figure 1) containing four dodecanucleotides each were obtained by separately coupling two hexamer pools (of two different 5'-terminal sequences each) with three different hexamer pools (of two different 3'-terminal sequences each).

D. Induction of fibroblasts

Human fibroblasts (cell line GM-2504A) were grown as described previously by Pestka et al., Proc. Natl. Acad. Sci. USA 72, 3898-3901 [1975]. Growth medium (Eagle's minimal essential medium containing 10% fetal calf serum) was removed from roller bottles (850 cm<sup>3</sup>) and replaced with 50 ml growth medium containing 50 µg/ml of poly(I):poly(C) and 10 µg/ml cycloheximide. This induction medium was removed after 4 hours at 37°C and cell monolayers were washed with phosphate buffered saline (PBS; 0.14M NaCl, 3mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 8mM Na<sub>2</sub>HPO<sub>4</sub>). Each bottle was incubated at 37°C with 10 ml of a trypsin - EDTA solution (Gibco 610-5305) until cells were detached, and fetal calf serum was added to a concentration of 10%. Cells were spun for 15 minutes at 500 x g and pellets were resuspended in PBS, pooled, and resedimented. Cells were frozen in liquid nitrogen. Approximately 0.17 g of cells were obtained per roller bottle.

20

E. Preparation and assay of interferon mRNA

Poly(A)-containing mRNA was prepared from human fibroblasts by phenol extractions and oligo(dT)-cellulose chromatography as described by Green et al. (Arch. Biochem. Biophys. 172, 74-89 [1975]). The poly (A) containing RNA was enriched for interferon mRNA by centrifugation on a linear 5-20% (w/v) sucrose gradient. The RNA samples were heated to 80°C for 2 minutes, rapidly cooled, layered over the gradient, and centrifuged for 20 hours at 30,000 rpm at 4°C in a Beckman SW-40 rotor. Fractions were collected, ethanol precipitated, and dissolved in H<sub>2</sub>O.

One microgram samples of mRNA were injected into *Xenopus laevis* oocytes as described by Cavalieri et al., Proc. Natl. Acad. Sci. USA 74, 3287-3291 [1977]. The injected oocytes were incubated 24 hours at 21°C, homogenized, and centrifuged for 5 minutes at 10,000 x g. The

interferon in the supernatant was determined by the cytopathic effect (CPE) inhibition assay (Stewart, The Interferon System, Springer-Verlag, New-York-Wien, 1979) using Sindbis virus and human diploid cells (WISH). Interferon  
5 titers of 1,000 to 6,000 units recovered (NIH reference standard) per microgram of RNA injected were routinely obtained for the 12S species of mRNA.

F. Synthesis and cloning of cDNA

10

Single stranded cDNA was prepared in 100  $\mu$ l reactions containing 5  $\mu$ g of 12S fraction mRNA, 20 mM Tris-HCl (pH 8.3), 20 mM KCl, 8mM  $MgCl_2$ , 30 mM  $\beta$ -mercaptoethanol, 100  $\mu$ Ci of ( $\alpha^{32}P$ )dCTP and 1 mM dATP, dCTP, dGTP, dTTP. The primer  
15 was the synthetic HindIII decamer dCCAAGCTTGG (Scheller et al., Science 196, 177-180 [1977]), which had been extended at the 3'-terminus with about 20 to 30 deoxythymidine residues using terminal deoxynucleotidyl transferase (Chang et al., Nature 275, 617-624 [1978]). 100 units  
20 of reverse transcriptase were added and the reaction mixture was incubated at 42°C for 30 minutes. The second strand DNA synthesis was carried out as described previously (Goeddel et al., Nature 281, 544-548 [1979]). The double stranded cDNA was treated with 1200 units of S1  
25 nuclease for 2 hours at 37°C in 25 mM sodium acetate (pH 4.5), 1mM  $ZnCl_2$ , 0.3M NaCl. After phenol extraction the mixture was separated electrophoretically on a 8% polyacrylamide gel. cDNA ( $\sim$  0.5  $\mu$ g) ranging from 550 to 1500 base pairs in size was recovered by electroelution. A 20 ng  
30 aliquot was extended with deoxyC residues using terminal deoxynucleotidyl transferase (Chang et al., supra), and annealed with 100 ng of pBR322 which had been cleaved with PstI and tailed with deoxyG residues (Chang et al., supra). The annealed mixture was used to transform E. coli K-12  
35 strain 294 by a published procedure (Hershfield et al., Proc. Natl. Acad. Sci. USA 71, 3455-3459 [1974]).

G. Preparation of induced and uninduced  $^{32}\text{P}$ -cDNA probes

5  $\mu\text{g}$  of 12S mRNA were combined with either 2  $\mu\text{g}$  of oligo (dT)<sub>12-18</sub> or 5  $\mu\text{g}$  of each synthetic primer pool (Figure 1) in 60  $\mu\text{l}$  of 10mM Tris-HCl (pH 8), 1 mM EDTA. The mixtures were boiled 3 minutes, and quenched on ice. 60  $\mu\text{l}$  of 40 mM Tris-HCl (pH 8.3), 40 mM KCl, 16mM  $\text{MgCl}_2$ , 60 mM  $\beta$ -mercaptoethanol, 1 mM dATP, dGTP, dTTP and  $5 \times 10^{-7}\text{M}$  ( $\alpha$ - $^{32}\text{P}$ ) dCTP (2,000 - 3,000 Ci/mM) was added to each template-primer mixture at 0°C. After the addition of 100 units of reverse transcriptase, the reactions were incubated at 42°C for 30 minutes and purified by passage over 10 ml Sephadex G-50 columns. The products were treated with 0.3N NaOH for 30 minutes at 70°C, neutralized, and ethanol precipitated.

The  $^{32}\text{P}$ -cDNAs were combined with 100.  $\mu\text{g}$  of poly(A) mRNA from uninduced fibroblasts in 50  $\mu\text{l}$  of 0.4M sodium phosphate (pH 6.8), 0.1% sodium dodecyl sulfate (SDS). The mixtures were heated at 98°C for 5 minutes and allowed to anneal 15 hours at 45°C. The DNA-RNA hybrids (containing uninduced cDNA sequences) were separated from single-stranded DNA (induced cDNA sequences) by chromatography on hydroxyapatite as described by Galau et al. (Proc. Natl. Acad. Sci. USA 74, 1020-1023 [1977]). The DNA-RNA hybrids were treated with alkali to remove RNA.

H. Screening of recombinant plasmids with  $^{32}\text{P}$ -cDNA probes

Approximately 1  $\mu\text{g}$  samples of plasmid DNA were prepared from individual transformants by a published procedure (Birnboim et al., Nucleic Acids Res. 7, 1513-1523 [1979]). The DNA samples were linearized by digestion with EcoRI, denatured in alkali, and applied to each of three nitrocellulose filters by the dot hybridization procedure (Kafatos et al., Nucleic Acids Res. 7; 1541-1552 [1979]). The filters were hybridized with the  $^{32}\text{P}$ -cDNA probes for 16 hours at 42°C in 50% formamide, 10x Denhardt's solution

(Biochem. Biophys. Res. Comm. 23, 641-646 [1966]), 6xSSC, 40 mM Tris-HCl (pH 7.5), 2mM EDTA, 40 µg/ml yeast RNA. Filters were washed with 0.1xSSC, 0.1% SDS twice for 30 minutes at 42°C, dried, and exposed to Kodak XR-2 x-ray film using Dupont Lightning-Plus intensifying screens at -80°C. [SSC contains 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0].

I. Construction of plasmids for direct expression of FIF

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The synthetic primers I (dATGAGCTACAAC) and II (dCATGAGCTACAAC) were phosphorylated using T4 polynucleotide kinase and ( $\gamma$ -<sup>32</sup>P)ATP to a specific activity of 700 Ci/mM as described by Goeddel et al., Proc. Natl. Acad. Sci. USA 76, 106-110 [1979]. Primer repair reactions were performed as follows: 250 pM of the <sup>32</sup>P-primers were combined with 8 µg (10 pM) of a 1200 bp HhaI restriction fragment containing the FIF cDNA sequence. The mixture was ethanol precipitated, resuspended in 50 µl H<sub>2</sub>O, boiled 3 minutes, quenched in a dry ice-ethanol bath, and combined with a 50 µl solution of 20mM Tris-HCl (pH 7.5), 14 mM MgCl<sub>2</sub>, 120 mM NaCl, 0.5 mM dATP, dCTP, dGTP, dTTP at 0°C. 10 units of DNA polymerase I Klenow fragment were added and the mixture was incubated at 37°C for 4 1/2 hours. Following extraction with phenol/CHCl<sub>3</sub> and restriction with PstI, the desired product was purified on a 6% polyacrylamide gel. Subsequent ligations were done at room temperature (cohesive termini) or 4°C (blunt ends) using conditions reported previously (Goeddel et al., supra).

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J. Assay for interferon expression in E. coli

Bacterial extracts were prepared for IF assay as follows: One ml cultures were grown overnight in LB (Luria-Bertani) medium containing 5 µg/ml tetracycline, then diluted into 25 ml of M9 medium supplemented with 0.2% glucose, 0.5% casamino acids and 5 µg/ml tetracycline. 10 ml samples were harvested by centrifugation when

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absorbance at 550 nm ( $A_{550}$ ) reached 1.0. The cell pellets were quickly frozen in a dry ice-ethanol bath and cleared lysates were prepared as described by Clewell (supra). Interferon activity in the supernatants was determined by comparison with NIH FIF standards using CPE inhibition assays. Two different assays were used: (a) WISH (human amnion) cells were seeded in microtiter dishes. Samples were added 16 to 20 hours later and diluted by serial 2-fold dilution. Sindbis virus was added after at least 3 hours of incubation. Plates were stained 20 to 24 hours later with crystal violet. (b) MDBK (bovine kidney) cell line was seeded simultaneously with 2-fold dilutions of samples. Vesicular stomatitis virus was added after 2 to 3 hours incubation and plates were stained with crystal violet 16 to 18 hours later. To test pH 2 stability bacterial extracts and standards were diluted in minimal essential medium to a concentration of 1000 units/ml. One ml aliquots were adjusted to pH 2 with 1N HCl, incubated at 4°C for 16 hours, and neutralized by addition of NaOH. IF activity was determined by the CPE inhibition assay using human amnion cells. To establish antigenic identity 25 µl aliquots of the 1000 U/ml interferon samples (untreated) were incubated with 25 µl of rabbit antihuman leukocyte interferon for 60 minutes at 37°C, centrifuged at 12,000 x g for 5 minutes and the supernatant assayed. Fibroblast and leukocyte interferon standards were obtained from the National Institutes of Health. Rabbit antihuman leukocyte interferon was obtained from the National Institute of Allergy and Infectious Diseases.

30

K. Chemical synthesis of primer pools complementary to FIF mRNA

The known amino-terminal protein sequence of human fibroblast interferon permitted us to deduce the 24 possible mRNA sequences which could code for the first four amino acids. The 24 complementary deoxyoligonucleotides were synthesized in 6 pools of 4 dodecamers each (Figure 1).

The six pools of 4 deoxyoligonucleotides each were synthesized by the modified phosphotriester method in solution and on solid phase (Crea et al., supra). The basic strategy involved reacting two different 3'-blocked trimers with an excess of a single 5'-protected trimer to yield a pool of two hexamers, each represented equally. The coupling of two pools, each containing two hexamers, then resulted in a pool of four dodecamers.

10 L. Identification of FIF cDNA clones

Using 12S mRNA from induced human fibroblasts (1,000 units IF activity per  $\mu\text{g}$  in oocyte assay), double stranded cDNA was prepared and inserted into pBR322 at the PstI site by the standard dG:dC tailing method as described by Chang et al., supra. A fibroblast cDNA library consisting of 30,000 ampicillin-sensitive, tetracycline-resistant transformants of E. coli K-12 strain 294 was obtained from 20 ng of cDNA ranging in size from 550 to 1300 base pairs. Plasmid DNA was prepared from 600 of the transformants and applied to 3 sets of nitrocellulose filters as described above.

The approach followed in the identification of hybrid plasmids containing fibroblast interferon cDNA sequences was similar to that used to identify human leukocyte interferon recombinant plasmids (Goeddel et al., Nature 287, 411-416 [1980]). Radiolabeled cDNA hybridization probes were prepared using either the 24 synthetic dodecamers or oligo(dT)<sub>12-18</sub> as primers and 12S RNA from induced fibroblasts (5000 units/ $\mu\text{g}$  in oocytes) as template. The <sup>32</sup>P-cDNAs (specific activity  $> 5 \times 10^8$  cpm/ $\mu\text{g}$ ) obtained were hybridized to a large excess of mRNA isolated from uninduced human fibroblasts, and the mRNA-cDNA hybrids were separated from unreacted cDNA by hydroxyapatite chromatography (Galau et al., supra). The single stranded cDNA fractions should be enriched for sequences which are present in induced fibroblasts but absent in uninduced

cells, and the mRNA-cDNA hybrids should represent sequences common to both induced and uninduced cells. Approximately  $4 \times 10^6$  cpm of single stranded cDNA (hybridization probe A) and  $8 \times 10^6$  cpm of cDNA-mRNA hybrids were obtained using  
5 oligo(dT)<sub>12-18</sub> primed cDNA;  $1.5 \times 10^6$  cpm of single stranded (hybridization probe B) and  $1.5 \times 10^6$  cpm of hybrids were obtained from cDNA primed using synthetic dodecamer pools 1-6. The cDNA-mRNA hybrids from both fractionations were combined, the RNA hydrolyzed by treatment  
10 with alkali, and the  $^{32}\text{P}$ -cDNA used as hybridization probe C. Many of the 600 plasmid samples hybridized with both probes A and C, indicating that the hybridization reactions between uninduced mRNA and  $^{32}\text{P}$ -cDNA (prior to the hydroxyapatite fractionation step) had not gone to completion. However, only one of the 600 plasmids (pF526) hybrid-  
15 ized strongly with the specifically primed, induced cDNA probe B (Figure 2). Plasmid pF526 also hybridized with the total oligo(dT)<sub>12-18</sub> primed, induced cDNA probe A, and failed to give detectable hybridization to the combined  
20 uninduced probe C.

PstI digestion of pF526 showed the cloned cDNA insert to be about 550 base pairs long, probably too short to contain the entire coding region for fibroblast inter-  
25 action. Therefore, a  $^{32}\text{P}$ -labeled DNA probe was prepared from this PstI fragment by random priming with calf thymus DNA (Taylor et al., supra). This probe was used to screen 2000 individual colonies from a newly constructed fibroblast cDNA library (the new cDNA library was prepared  
30 using 12S mRNA from induced fibroblasts having a titer of 6,000 units/ml in the oocyte assay system). Sixteen clones hybridized to the probe. Plasmids prepared from the majority of these released two fragments when cleaved with PstI, indicating that the cDNA contained an internal  
35 PstI site. Clone pFIF3 contained the largest cDNA insert, about 800 base pairs. The DNA sequence of the insert was determined by the Maxam-Gilbert procedure (supra) and is shown in Figure 3. The amino acid sequence of human fibro-



blast interferon predicted from the nucleotide sequence is identical to that reported recently by Taniguchi et al. (Gene 10, 11-15 [1980]) and by Derynck et al. (supra) from DNA sequencing of FIF cDNA clones. A precursor or  
5 signal peptide of 21 amino acids is followed by a sequence of 166 amino acids representing the mature interferon, a stretch of 196 3'-untranslated nucleotides and a poly(A) tail. The NH<sub>2</sub>-terminal 20 amino acids of mature FIF have been directly determined by protein microsequencing and  
10 are the same as those predicted from the DNA sequence.

#### M. Direct expression of fibroblast interferon

To express high levels of mature fibroblast interferon in E. coli initiation of protein synthesis must  
15 occur at the ATG codon of the mature polypeptide (amino acid 1) rather than at the ATG of the signal peptide (amino acid S1) (Figure 3).

Our approach to removing the signal peptide coding regions from pFIF3 is depicted in Figure 4. A 1200 bp DNA fragment which contained the entire cDNA insert was isolated from a polyacrylamide gel after digesting pFIF3 with HhaI. Two separate synthetic deoxyoligonucleotide  
20 primers, dATGAGCTACAAC(I) and dCATGAGCTACAAC(II), were prepared. Both primers contain the coding sequence for the first four amino acids of mature fibroblast interferon; primer II has an additional C at the 5'-terminus. Primer repair reactions and subsequent ligations were  
25 carried out separately for primers I and II, and gave nearly identical results. Therefore, only reactions using primer I are discussed in detail here. The primers were 5'-radiolabeled using ( $\gamma$ -<sup>32</sup>P)ATP and T4 polynucleotide kinase, combined with the 1200 bp HhaI DNA fragment and  
30 the mixtures were denatured by boiling. Following hybridization of the primer to the denatured HhaI DNA fragment, E. coli DNA polymerase I Klenow fragment (Klenow et al.,  
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Proc. Natl. Acad. Sci. USA 65, 168-175 [1970]) was used to catalyze the repair synthesis of the plus (top) strand (Figure 4). In addition, the associated 3' → 5' exonuclease activity of the Klenow fragment removed the 3'-  
5 protruding end from the minus (bottom) strand, leaving a flush end. Analysis of samples of the reaction mixture by polyacrylamide gel electrophoresis indicated that the repair synthesis did not go to completion, but stopped at several discrete sites. Therefore, the entire reaction  
10 mixture was treated with PstI and the desired 141 bp fragment (180,000 Cerenkov cpm; ~0.3 pM) was purified by polyacrylamide gel electrophoresis (Figure 5). Ligation of this fragment to 1 µg (~4 pM) of the 363 bp PstI-BglIII fragment isolated from pFIF3 (Fig. 4), followed by BglIII  
15 digestion, yielded 50,000 Cerenkov cpm (~0.1 pM, ~30 ng) of the 504 bp DNA fragment containing the entire coding sequence for mature fibroblast interferon. The same reactions using primer II gave 83,000 cpm (~0.15 pM, ~50 ng) of 505 bp product.

20

The construction of plasmids which direct the synthesis of human fibroblast interferon is outlined in Figure 6. Separate expression plasmids were constructed which placed FIF synthesis under the control of the *E. coli*  
25 lac or trp promoter-operator systems. Both of these systems have proven useful for the direct expression of eukaryotic genes in *E. coli*: human growth hormone has been efficiently synthesized using the lac system (Goeddel et al., Nature 281, 544-548 [1979]) and human leukocyte interferon has been produced at high levels using the trp  
30 system (Goeddel et al., Nature 287, 411 [1980]).

pBRH trp was digested with EcoRI restriction enzyme and the resulting fragment isolated by PAGE and electro-  
35 elution. EcoRI-digested plasmid pSom 11 (Itakura et al., Science 198, 1056-1063 [1977]); G.B. patent publication no. 2 007 676 A) was combined with the above fragment. The mixture was ligated with T<sub>4</sub> DNA ligase and the resul-

ting DNA transformed into E. coli K-12 strain 294 as previously described. Transformant bacteria were selected on ampicillin-containing plates. Resulting ampicillin-resistant colonies were screened by colony hybridization (Grunstein et al., supra) using as a probe the trp promoter-operator containing the above fragment isolated from pBRHtrp, which had been radioactively labelled with P<sup>32</sup>. Several colonies shown positive by colony hybridization were selected, plasmid DNA was isolated and the orientation of the inserted fragments determined by restriction analysis employing restriction enzymes BglII and BamHI in double digestion. E. coli 294 containing the plasmid designated pSOM7Δ2, which has the trp promoter-operator fragment in the desired orientation was grown in LB medium containing 10 µg/ml ampicillin. The cells were grown to optical density 1 (at 550 nm), collected by centrifugation and resuspended in M9 media in tenfold dilution. Cells were grown for 2-3 hours, again to optical density 1, then lysed and total cellular protein analyzed by SDS urea (15%) PAGE (Maizel et al., Methods Virol. 5, 180-246 [1971]).

Plasmid pBR322 was HindIII digested and the protruding HindIII ends in turn digested with S1 nuclease. The S1 nuclease digestion involved treatment of 10 µg of HindIII-cleaved pBR322 in 30 µl S1 buffer (0.3 M NaCl, 1 mM ZnCl<sub>2</sub>, 25 mM sodium acetate, pH 4.5) with 300 units S1 nuclease for 30 minutes at 15°C. The reaction was stopped by the addition of 1 µl of 30 x S1 nuclease stop solution (0.8M Tris base, 50 mM EDTA). The mixture was phenol extracted, chloroform extracted and ethanol precipitated, then EcoRI digested as previously described and the large fragment (1) obtained by PAGE procedure followed by electroelution. The fragment obtained has a first EcoRI sticky end and a second, blunt end whose coding strand begins with the nucleotide thymidine.

Plasmid pSom7Δ2, as prepared above, was BglII digested and the BglII sticky ends resulting made double stranded with the Klenow polymerase I procedure using all four deoxynucleotide triphosphates. EcoRI cleavage of the resulting product followed by PAGE and electroelution of the small fragment (2) yielded a linear piece of DNA containing the tryptophan promoter-operator and codons of the LE' "proximal" sequence upstream from the BglII site ("LE'(p)"). The product had an EcoRI end and a blunt end resulting from filling in the BglII site. However, the BglII site is reconstituted by ligation of the blunt end of fragment (2) to the blunt end of fragment (1). Thus, the two fragments were ligated in the presence of T<sub>4</sub> DNA ligase to form the recirculated plasmid pHKY 10 which was propagated by transformation into competent E. coli strain 294 cells.

Plasmid pGM1 carries the E. coli tryptophan operon containing the deletion ΔLE1413 (Miozzari et al., J. Bacteriology 133, 1457-1466 [1978]) and hence expresses a fusion protein comprising the first 6 amino acids of the trp leader and approximately the last third of the trp E polypeptide (hereinafter referred to in conjunction as LE'), as well as the trp D polypeptide in its entirety, all under the control of the trp promoter-operator system. The plasmid, 20 μg, was digested with the restriction enzyme PvuII which cleaves the plasmid at five sites. The gene fragments were next combined with EcoRI linkers (consisting of a self complementary oligonucleotide of the sequence: pCATGAATTCATG) providing an EcoRI cleavage site for a later cloning into a plasmid containing an EcoRI site. The 20 μg of DNA fragments obtained from pGM1 were treated with 10 units T<sub>4</sub> DNA ligase in the presence of 200 pico moles of the 5'-phosphorylated synthetic oligonucleotide pCATGAATTCATG and in 20 μl T<sub>4</sub> DNA ligase buffer (20mM Tris, pH 7.6, 0.5 mM ATP, 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol) at 4°C overnight. The solution was then heated 10 minutes at 70°C to halt ligation. The linkers

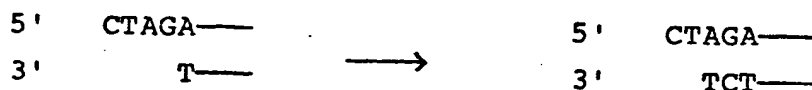
were cleaved by EcoRI digestion and the fragments, now with EcoRI ends were separated using 5% PAGE and the three largest fragments isolated from the gel by first staining with ethidium bromide, locating the fragments with ultra-violet light, and cutting from the gel the portions of interest. Each gel fragment, with 300 microliters 0.1xTBE, was placed in a dialysis bag and subjected to electrophoresis at 100 V for one hour in 0.1xTBE buffer (TBE buffer contains: 10.8 gm Tris base, 5.5 gm boric acid, 0.09 gm Na<sub>2</sub>EDTA in 1 liter H<sub>2</sub>O). The aqueous solution was collected from the dialysis bag, phenol extracted, chloroform extracted and made 0.2 M sodium chloride, and the DNA recovered in water after ethanol precipitation. The trp promoter-operator containing gene with EcoRI sticky ends was identified in the procedure next described, which entails the insertion of fragments into a tetracycline sensitive plasmid which, upon promoter-operator insertion, becomes tetracycline resistant.

Plasmid pBRH1 (Rodriguez et al., Nucleic Acids Research 6, 3267-3287 [1979]) expressed ampicillin resistance and contains the gene for tetracycline resistance but, there being no associated promoter, does not express that resistance. The plasmid is accordingly tetracycline sensitive. By introducing a promoter-operator system in the EcoRI site, the plasmid can be made tetracycline resistant.

pBRH1 was digested with EcoRI and the enzyme removed by phenol extraction followed by chloroform extraction and recovered in water after ethanol precipitation. The resulting DNA molecule was, in separate reaction mixtures, combined with each of the three DNA fragments obtained above and ligated with T<sub>4</sub> DNA ligase as previously described. The DNA present in the reaction mixture was used to transform competent E. coli K-12 strain 294 by standard techniques (Hershfield et al., supra) and the bacteria

plated on LB plates containing 20 µg/ml ampicillin and 5 µg/ml tetracycline. Several tetracycline-resistant colonies were selected, plasmid DNA isolated and the presence of the desired fragment confirmed by restriction enzyme analysis. The resulting plasmid is designated pBRHtrp.

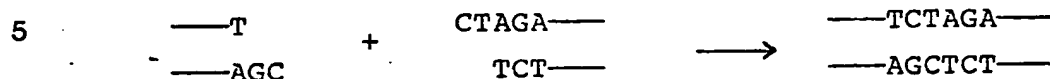
An EcoRI and BamHI digestion product of the viral genome of hepatitis B was obtained by conventional means and cloned into the EcoRI and BamHI sites of plasmid pGH6 (Goeddel et al., Nature 281, 544-548 [1979]) to form the plasmid pHS32. Plasmid pHS32 was cleaved with XbaI, phenol extracted, chloroform extracted and ethanol precipitated. It was then treated with 1 µl E. coli polymerase I, Klenow fragment, in 30 µl polymerase buffer (50 mM potassium phosphate pH 7.4, 7mM MgCl<sub>2</sub>, 1 mM β-mercaptoethanol) containing 0.1mM dTTP and 0.1mM dCTP for 30 minutes at 0°C then 2 hours at 37°C. This treatment causes 2 of the 4 nucleotides complementary to the 5' protruding end of the XbaI cleavage site to be filled in:



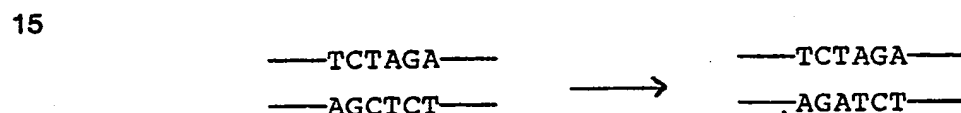
Two nucleotides, dC and dT, were incorporated giving an end with two 5' protruding nucleotides. This linear residue of plasmid pHS32 (after phenol and chloroform extraction and recovery in water after ethanol precipitation) was cleaved with EcoRI. The large plasmid fragment was separated from the smaller EcoRI-XbaI fragment by PAGE and isolated after electroelution. This DNA fragment from pHS32 (0.2 µg) was ligated under conditions similar to those described above, to the EcoRI-Taq I fragment of the tryptophan operon (~0.01 µg), derived from pBRHtrp.

In the process of ligating the fragment from pHS32 to the EcoRI-TaqI fragment, as described above, the

TaqI protruding end is ligated to the XbaI remaining protruding end even though it is not completely Watson-Crick base-paired:



A portion of this ligation reaction mixture was transformed into E. coli 294 cells, heat treated and plated on LB plates containing ampicillin. Twenty-four colonies were selected, grown in 3 ml LB media, and plasmid isolated. Six of these were found to have the XbaI site regenerated via E. coli catalyzed DNA repair and replication.



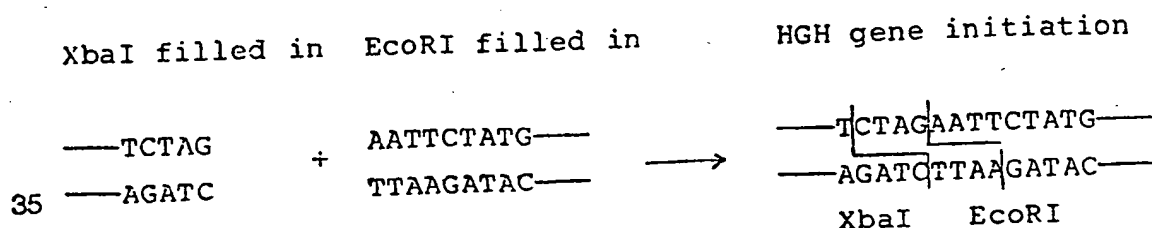
These plasmids were also found to cleave both with EcoRI and HpaI and to give the expected restriction fragments. One plasmid, designated pTrp14, was used for expression of heterologous polypeptides, as next discussed.

The plasmid pGH 107 (Goeddel et al., Nature 281, 544-548 [1979]) contains a gene for human growth hormone made up of 23 amino acid codons produced from synthetic DNA fragments and 163 amino acid codons obtained from complementary DNA produced via reverse transcription of human growth hormone messenger RNA. This gene, though it lacks the codons of the "pre" sequence of human growth hormone, does contain an ATG translation initiation codon. The gene was isolated from 10 µg pGH 107 after treatment with EcoRI followed by E. coli polymerase I Klenow fragment and dTTP and dATP as described above. Following phenol and chloroform extraction and ethanol precipitation the plasmid was treated with BamHI.

The human growth hormone (HGH) gene-containing fragment was isolated by PAGE followed by electroelution. The resulting DNA fragment also contains the first 350 nucleotides of the tetracycline resistance structural gene, but lacks the tetracycline promoter-operator system so that, when subsequently cloned into an expression plasmid, plasmids containing the insert can be located by the restoration of tetracycline resistance. Because the EcoRI end of the fragment has been filled in by the Klenow polymerase I procedure, the fragment has one blunt and one sticky end, ensuring proper orientation when later inserted into an expression plasmid.

The expression plasmid pTrp14 was next prepared to receive the HGH gene-containing fragment prepared above. Thus, pTrp14 was XbaI digested and the resulting sticky ends filled in with the Klenow polymerase I procedure employing dATP, dTTP, dGTP and dCTP. After phenol and chloroform extraction and ethanol precipitation the resulting DNA was treated with BamHI and the resulting large plasmid fragment isolated by PAGE and electroelution. The pTrp14-derived fragment had one blunt and one sticky end, permitting recombination in proper orientation with the HGH gene containing fragment previously described.

The HGH gene fragment and the pTRP14  $\Delta$ Xba-BamHI fragment were combined and ligated together under conditions similar to those described above. The filled in XbaI and EcoRI ends ligated together by blunt end ligation to recreate both the XbaI and the EcoRI site:





This construction also recreates the tetracycline resistance gene. Since the plasmid pGH 107 expresses tetracycline resistance from a promoter lying upstream from the HGH gene (the lac promoter), this construction, 5 designated pGH 207, permits expression of the gene for tetracycline resistance under the control of the tryptophan promoter-operator. Thus the ligation mixture was transformed into E. coli 294 and colonies selected on LB plates containing 5 µg/ml tetracycline.

10

Plasmid pGH 207 was EcoRI digested and the trp promoter containing EcoRI fragment recovered by PAGE followed by electroelution. Plasmid pBRH1 was EcoRI digested and the cleaved ends treated with bacterial alkaline phosphatase (BAP, 1 µg, in 50 mM Tris, pH 8, and 10 mM MgCl<sub>2</sub> 15 for 30 min. at 65°C) to remove the phosphate groups on the protruding EcoRI ends. Excess bacterial alkaline phosphatase was removed by phenol extraction, chloroform extraction and ethanol precipitation. The resulting linear 20 DNA, because it lacks phosphates on the protruding ends thereof, will in ligation accept only inserts whose complementary sticky ends are phosphorylated but will not itself recircularize, permitting more facile screening for plasmids containing the inserts.

25

The EcoRI fragment derived from pGH 207 and the linear DNA obtained from pBRH1 were combined in the presence of T<sub>4</sub> ligase as previously described and ligated. A portion of the resulting mixture was transformed into 30 E. coli strain 294 as previously described, plated on LB media containing 5 µg/ml of tetracycline, and 12 tetracycline resistant colonies selected. Plasmid was isolated from each colony and examined for the presence of a DNA insert by restriction endonuclease analysis employing 35 EcoRI and XbaI. One plasmid containing the insert was designated pHKY1.

The plasmid pHKY10, described above, is a derivative of pBR322 which contains a BglII site between the tetracycline resistance ( $Tc^R$ ) promoter and structural gene. The large DNA fragment isolated after digesting pHKY10 with PstI and BglII therefore contains part of the ampicillin resistance ( $Ap^R$ ) gene and all of the  $Tc^R$  structural gene, but lacks the  $Tc^R$  promoter (Fig. 6). The plasmid pGH6 (Goeddel et al., Nature 281, 544-548 [1979]) was digested with EcoRI, the resulting single stranded ends were filled in with DNA polymerase I, and the plasmid was cleaved with PstI. The small fragment, containing part of the  $Ap^R$  gene, a double lac promoter and lac ribosome binding site, but lacking an ATG initiation triplet was isolated. A similar trp promoter fragment, containing the trp leader ribosome binding site, but lacking an ATG sequence (Goeddel et al., Nature 287, 411-416 [1980]), may be isolated from pHKY1 described above.

The trp fragment just referred to is an analog of the E. coli tryptophan operon from which the so-called trp attenuator has been deleted (Miozzari et al., J. Bact. 133, 1457-1466 [1978]) to controllably heighten expression levels. Expression plasmids containing the modified trp regulon can be grown to predetermined levels in nutrient media containing additive tryptophan in quantities sufficient to repress the promoter-operator system, then be deprived of tryptophan so as to derepress the system and occasion the expression of the intended product.

The expression plasmids may be assembled via three part ligation reactions as shown in Figure 6. 15 ng ( 0.05 pM) of the assembled FIF gene (504 or 505 bp), 0.5  $\mu$ g ( 0.2 pM) of the large PstI - BglII fragment of pHKY10 and 0.2  $\mu$ g ( 0.3 pM) of the appropriate promoter fragment were ligated and the mixture used to transform E. coli 294 (Goeddel et al., Nature 287, 411-416 [1980]). Plasmid DNA was prepared from individual transformants and analyzed by restriction mapping. Correct joining of the assembled gene to the promoter fragment should restore

the EcoRI (lac) or the XbaI (trp) recognition sequences. The majority of the plasmids gave the expected restriction enzyme digestion patterns. Individual clones (12 containing the trp promoter and 12 containing the lac promoter) were  
5 grown and extracts prepared for interferon assay as described above.

When assayed on human amnion (WISH) cells for anti-viral activity by the CPE inhibition assay five of the trp  
10 transformants were positive (each approximately equivalent); eleven of the lac transformants gave equivalent IF activities. Therefore, one transformant from each series (pFIFlac9 and pFIFtrp69) was selected for further study (Table 1). DNA sequence analysis demonstrated that the  
15 desired attachment of promoter to FIF structural gene had occurred in both cases.

Table 1. Interferon activity in extracts of E. coli

20	E. coli K-12 strain 294 transformed by	Cell density (cells/ml)	IF Activity (units/l culture)	FIF molecules per cell
25	pBR322	$3.5 \times 10^8$	-	-
	pFIFlac9	$3.5 \times 10^8$	$9.0 \times 10^6$	2,250
	pFIFtrp69	$3.5 \times 10^8$	$1.8 \times 10^7$	4,500
	pFIFtrp <sup>3</sup> 69	$3.5 \times 10^8$	$8.1 \times 10^7$	20,200

30 Cells were grown and extracts prepared as described above. The human amnion (WISH) cell line was used for the CPE inhibition assay. Activities given are the average from three independent experiments. To determine the number of IF molecules per cell a FIF specific activity  
35 of  $4 \times 10^8$  units/mg was used (Knight, supra).

The amounts of fibroblast interferon produced by pFIFlac9 and pFIFtrp69 are shown in Table 1. The trp

promoter gave a FIF expression level measurable higher than did the lac promoter. In an attempt to further increase FIF expression levels, pFIFtrp69 was cleaved with EcoRI and two 300 base pair EcoRI fragments containing the trp promoter (Goeddel et al., Nature 287, 411-416 [1980]) were inserted. The resulting plasmid, pFIFtrp<sup>3</sup>69, contains three successive trp promoters which read toward the FIF gene. The amount of FIF synthesized by E. coli K-12 strain 294/pFIF trp<sup>3</sup>69 is 4-5 times that produced by pFIFtrp69 (Table 1). This is apparently due to the derepression of the trp promoter which occurs when trp repressor levels are titrated by the multiple copies of the trp operator.

The FIF produced by E. coli K-12 strain 294/pFIFtrp69 behaves like authentic human FIF. As shown in Table 2, its antiviral activity is about 30 times greater on human cells than on bovine cells. In addition, the bacterially produced FIF is stable to treatment at pH 2 overnight and is not neutralized by rabbit antihuman leukocyte interferon antibodies (Table 3).

Table 2. Interferon activities measured on different cell types

Cells	Interferon Activity (units/ml)		
	LeIF	FIF	E. coli K-12 strain 294/pFIFtrp69 extract
Human amnion	20,000	10,000	1280
Bovine kidney	13,000	400	40

LeIF and FIF were NIH standard solutions having 20,000 units/ml and 10,000 units/ml respectively. Assays were performed as described above.

Table 3. Comparison of activities of extracts from E. coli K-12 strain 294/pFIFtrp69 with standard human leukocyte and fibroblast interferons

5

	Interferon Activity (units/ml)		
	LeIF	FIF	E. coli K-12 strain 294/pFIFtrp69
10 untreated	1000	1000	1000
pH 2	1000	1000	1000
rabbit antihuman	<16	1000	1000
LeIF antibodies			

15

Experimental procedures described above. Assayed by CPE inhibition using WISH cells/Sindbis virus.

N. Purification

20

The purification procedure for bacterial derived fibroblast interferon is as follows:

1. Frozen cells are suspended in twelve times volume per weight with sucrose lysis buffer (100mM Tris-HCl, 10% sucrose, 0.2M NaCl, 50mM EDTA, 0.2mM PMSF [phenylmethylsulfonyl chloride], pH 7.9) containing lysozyme at 1mg/ml. The cell suspension is stirred for 1 hour at 4°C and centrifuged. Fibroblast interferon activity remains in the supernatant.

2. Polyethyleneimine (5%, v/v) is added to the sonicated supernatant to a final concentration of 0.5% (v/v). The solution is stirred for 1 hour at 4°C and centrifuged. Interferon activity remains in the supernatant.

3. Solid ammonium sulfate is added to the polyethyleneimine supernatant to a final concentration of 50% saturation, stirred for 30 minutes at 4°C and centrifuged. Interferon activity is in the 50% pellet.

5

4. The 50% ammonium sulfate pellet is suspended in one half the volume of the 50% ammonium sulfate suspension with PBS (20 mM sodium phosphate, 0.15M NaCl, pH 7.4). Polyethylene glycol 6000 (50%, w/v, in PBS) is added to a  
10 final concentration of 12.5% (v/v), stirred at 4°C for 2 hours and centrifuged. Interferon activity is in the pellet. The pellet is suspended in a minimal volume of sucrose lysis buffer and clarified by centrifugation.

15

This initial extraction procedure results in a purification of fibroblast interferon from 0.001% of the total protein to 0.05% of the total protein. This material can be further purified to homogeneity by the following column chromatography steps:

20

5. Affinity chromatography on Amicon Blue B in sucrose lysis buffer.

6. Anion exchange chromatography on QAE Sephadex in  
25 sucrose lysis buffer in the absence of 0.2M NaCl.

7. Size exclusion chromatography on Sephadex G-75 in sucrose lysis buffer.

30 8. Reverse phase high pressure liquid chromatography.

0. Parenteral Administration

FIF may be parenterally administered to subjects  
35 requiring antitumor or antiviral treatment. Dosage and dose rate may parallel that currently in use in clinical investigations of human derived materials, e.g., about  $(1-10) \times 10^6$  units daily, and in the case of materials of

purity greater than 1%, likely up to, e.g.,  $15 \times 10^7$  units daily. Dosages of bacterially obtained FIF could be significantly elevated for greater effect owing to the essential absence of human proteins other than FIF, which proteins in fibroblast-derived materials may act as pyrogens, exhibiting adverse effects, e.g., malaise, temperature elevation, etc.

As one example of an appropriate dosage form for essentially homogeneous bacterial FIF in parenteral form, 3 mg FIF of specific activity of, say,  $2 \times 10^8$  U/mg may be dissolved in 25 ml of 5% human serum albumin, the solution is passed through a bacteriological filter and the filtered solution aseptically subdivided into 100 vials, each containing  $6 \times 10^6$  units pure interferon suitable for parenteral administration. The vials are preferably stored in the cold ( $-20^\circ\text{C}$ ) prior to use.

The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby the polypeptide hereof is combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation are described in Remington's Pharmaceutical Sciences by E.W. Martin, which is hereby incorporated by reference. Such compositions will contain an effective amount of the interferon protein hereof together with a suitable amount of vehicle in order to prepare pharmaceutically acceptable compositions suitable for effective administration to the host. One preferred mode of administration is parenteral.

What we claim is:

1. A polypeptide comprising the amino acid sequence of a mature human fibroblast interferon, microbially produced and unaccompanied by any corresponding presequence or portion thereof.

2. A polypeptide according to claim 1, unaccompanied by associated glycosylation.

3. The polypeptide according to claim 1, optionally containing the amino acid methionine as the ordinarily first amino acid of said interferon.

4. The polypeptide according to claim 1, optionally containing a cleavable conjugate or microbial signal protein attached to the N-terminus of the ordinarily first amino acid of said interferon.

5. A DNA sequence comprising a sequence coding for the polypeptide according to claims 1, 3 or 4.

6. The DNA sequence according to claim 5 operably linked with a DNA sequence capable of effecting microbial expression of a polypeptide according to claims 1, 3 or 4.

7. A replicable microbial expression vehicle capable, in a transformant microorganism, of expressing a polypeptide according to claims 1, 3 or 4.

8. A microbial expression vehicle according to claim 7 which is a plasmid.

9. A plasmid selected from the group consisting of pFIFlac9, pFIFtrp69, and pFIFtrp<sup>3</sup>69.

10. A microorganism transformed with an expression vehicle according to any one of claims 7-9.



11. The microorganism according to claim 10, obtained by transforming an *E. coli* strain.

12. A transformed microorganism according to claim 11  
5 wherein said *E. coli* strain is *E. coli* K-12 strain 294.

13. A transformed microorganism according to claim 10 obtained by transforming *Bacillus subtilis*.

10 14. A transformed microorganism according to claim 10 obtained by transforming *Saccharomyces cerevisiae*.

15 15. A composition of matter comprising a therapeutically active fraction of a polypeptide consisting essentially of the amino acid sequence of a mature human fibroblast interferon, the balance of said composition comprising soluble microbial protein from which said polypeptide may be purified to a degree sufficient for effective therapeutic application.

20

16. A bacterial extract comprising greater than about 95% pure polypeptide consisting essentially of the amino acid sequence of a mature fibroblast interferon according to any one of claims 1-4.

25

17. A pharmaceutical composition comprising a therapeutically effective amount of a mature human fibroblast interferon according to claims 1, 3 or 4, suitable for pharmaceutical administration.

30

18. The composition according to claim 17 suitable for parenteral administration.

19. A culture of microbial cells capable of producing  
35 a human fibroblast interferon in mature form.

20. The use of a mature human fibroblast interferon according to claims 1, 3 or 4, for antitumor or antiviral treatment or for preparing pharmaceutical compositions useful for such treatment.

5

21. A process for producing a polypeptide claimed in any one of claims 1-4 which process comprises causing a microorganism, transformed with a replicable microbial expression vehicle capable of expressing said polypeptide, 10 to grow up and express said polypeptide and recovering it.

22. A process for producing microorganisms capable of expressing a polypeptide claimed in any one of claims 1-4 which process comprises transforming a microorganism 15 with a replicable microbial expression vehicle capable of expressing said polypeptide and cultivating the transformed microorganism.

23. A process for producing a replicable microbial 20 expression vehicle capable in a transformant microorganism of expressing a polypeptide as claimed in any one of claims 1-4, which process comprises constructing a first DNA sequence coding for said polypeptide and operably linking said first DNA sequence with a second DNA sequence 25 capable of effecting microbial expression of said first DNA sequence.

24. The process of claim 23 wherein said second DNA sequence comprises a multiple trp-promoter-operator. 30

25. The products and processes for their preparation as hereinbefore described.

\*\*\*

35

What we claim is: A U S P A T E N T

1. A process for producing a polypeptide comprising  
the-amino acid sequence of a mature human fibroblast  
5 interferon unaccompanied by any corresponding presequence  
or portion thereof, which process comprises causing a micro-  
organism transformed with a replicable microbial ex-  
pression vehicle capable of expressing said polypeptide,  
to grow up and to express said polypeptide and recovering  
10 it.

2. A process as claimed in claim 1, wherein the  
polypeptide is unaccompanied by associated glycosylation.

15 3. A process as claimed in claim 1, wherein the poly-  
peptide optionally contains the amino acid methionine as  
the ordinarily first amino acid of said fibroblast inter-  
feron.

20 4. A process as claimed in claim 1, wherein the poly-  
peptide optionally contains a cleavable conjugate or micro-  
bial signal protein attached to the N-terminus of the  
ordinarily first amino acid of said fibroblast interferon.

25 5. A process for the production of microorganisms  
capable of producing a polypeptide comprising the amino  
acid sequence of a mature human fibroblast interferon  
unaccompanied by any corresponding presequence or portion  
thereof, which process comprises transforming a micro-  
30 organism with a replicable microbial expression vehicle  
capable of expressing said polypeptide and cultivating  
the transformed microorganism.

35 6. A process for the production of a replicable  
microbial expression vehicle capable in a transformant  
microorganism of expressing a polypeptide comprising the  
amino acid sequence of a mature human fibroblast interferon  
unaccompanied by any corresponding presequence or portion

thereof which process comprises constructing a first DNA sequence coding for said polypeptide and operably linking said first DNA sequence with a second DNA sequence capable of effecting microbial expression of said first DNA  
5 sequence.

7. The process of claim 6 wherein said second DNA sequence comprises a multiple trp promoter-operator.

10 8. A process for the preparation of pharmaceutical compositions containing a microbially produced polypeptide comprising the amino acid sequence of a mature human fibroblast interferon unaccompanied by any corresponding presequence or portion thereof which process comprises  
15 mixing said polypeptide with non-toxic, inert, therapeutically compatible carriers and bringing the resulting mixture into a suitable pharmaceutical dosage form.

20 9. A pharmaceutical composition containing a microbially produced polypeptide comprising the amino acid sequence of a mature human fibroblast interferon unaccompanied by any corresponding presequence or portion thereof and a non-toxic, inert, therapeutically compatible carrier material.

25 10. The processes for the preparation of polypeptides, replicable microbial expression vehicles and microorganisms as hereinbefore described.

30 11. A DNA sequence comprising a sequence coding for the amino acid sequence of a mature human fibroblast interferon unaccompanied by any corresponding presequence or portion thereof.

35 12. A DNA sequence comprising a sequence coding for the amino acid sequence of a mature human fibroblast interferon the amino acid methionine optionally being the ordinarily first amino acid of said interferon.

13. A DNA sequence comprising a sequence coding for the amino acid sequence of a mature human fibroblast interferon as well as for the amino acid sequence of a cleavable conjugate or microbial signal protein attached to the N-terminus of the ordinarily first amino acid of said interferon.

14. A DNA sequence according to any one of claims 11-13 operably linked with a DNA sequence capable of effecting microbial expression of the encoded polypeptide.

15. A replicable microbial expression vehicle capable, in a transformant microorganism, of expressing a polypeptide coded by a DNA sequence according to any one of claims 11-14.

16. An expression vehicle as claimed in claim 15 which is a plasmid.

17. A plasmid selected from the group consisting of pFIFlac9, pFIFtrp69 and pFIFtrp<sup>3</sup>69.

18. A microorganism transformed with an expression vehicle according to any one of claims 15-17.

19. The microorganism according to claim 18 obtained by transforming an E. coli strain.

20. A transformed microorganism according to claim 19 wherein said E. coli strain is E. coli K-12 strain 294.

21. A transformed microorganism according to claim 18 obtained by transforming Bacillus subtilis.

22. A transformed microorganism according to claim 18 obtained by transforming Saccharomyces cerevisiae.

Protein1 2 3 4  
Met-Ser-Tyr-Asn-mRNA(5') AUG-UC<sup>G</sup><sub>A</sub>-UA<sup>U</sup><sub>C</sub>-AA<sup>U</sup><sub>C</sub> (16 combinations)(5') AUG-AG<sup>U</sup><sub>C</sub>-UA<sup>U</sup><sub>C</sub>-AA<sup>C</sup><sub>U</sub> (8 combinations)Complementary DNA primersATT-<sup>A</sup><sub>G</sub>TA-<sup>T</sup><sub>C</sub>GA-CAT Pool 1ATT-<sup>A</sup><sub>G</sub>TA-<sup>A</sup><sub>G</sub>GA-CAT Pool 2ATT-<sup>A</sup><sub>G</sub>TA-<sup>A</sup><sub>G</sub>CT-CAT Pool 3GTT-<sup>A</sup><sub>G</sub>TA-<sup>T</sup><sub>C</sub>GA-CAT Pool 4GTT-<sup>A</sup><sub>G</sub>TA-<sup>A</sup><sub>G</sub>GA-CAT Pool 5GTT-<sup>A</sup><sub>G</sub>TA-<sup>A</sup><sub>G</sub>CT-CAT Pool 6*FIG. 1.*

*[The page contains several faint circular stamps, likely from various government departments or offices.]*

*[The page contains extremely faint, illegible markings.]*

501 510 520  
530 540 550

FIG. 2.

5'  
 S1  
 met thr asn lys cys leu leu gln ile ala leu leu leu cys phe ser thr thr ala leu ser MET SER TYR ASN  
 ATG ACC AAC AAG TGT CTC CTC CAA ATT GCT CTC CTG TTG TGC TTC TCC ACT ACA GCT CTT TCC ATG AGC TAC AAC  
 50  
 10  
 LEU LEU GLY PHE LEU GLN ARG SER SER ASN PHE GLN CYS GLN LYS LEU LEU TRP GLN LEU ASN GLY ARG LEU GLU  
 TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT CAG TGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA  
 100  
 20  
 LEU LEU TRP GLN LEU ASN GLY ARG LEU GLU  
 TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT CAG TGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA  
 150  
 30  
 TYR CYS LEU LYS ASP ARG MET ASN PHE ASP 40  
 TAT TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG CAG CTG CAG CAG TTC CAG AAG GAG GAC  
 200  
 50  
 TYR CYS LEU LYS ASP ARG MET ASN PHE ASP 40  
 TAT TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG CAG CTG CAG CAG TTC CAG AAG GAG GAC  
 250  
 60  
 ALA ALA LEU THR ILE TYR GLU MET LEU GLN ASN ILE PHE ALA ILE PHE ARG GLN ASP SER SER SER THR GLY TRP  
 GCC GCA TTG ACC ATC TAT GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT AGC ACT GGC TGG  
 250  
 70  
 ALA ALA LEU THR ILE TYR GLU MET LEU GLN ASN ILE PHE ALA ILE PHE ARG GLN ASP SER SER SER THR GLY TRP  
 GCC GCA TTG ACC ATC TAT GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT AGC ACT GGC TGG  
 300  
 80  
 ASN GLU THR ILE VAL GLU ASN LEU LEU ALA ASN VAL TYR HIS GLN ILE ASN HIS LEU LYS THR VAL LEU GLU GLU  
 AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA GTC CTG GAA GAA  
 350  
 90  
 ASN GLU THR ILE VAL GLU ASN LEU LEU ALA ASN VAL TYR HIS GLN ILE ASN HIS LEU LYS THR VAL LEU GLU GLU  
 AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA GTC CTG GAA GAA  
 400  
 110  
 LYS LEU GLU LYS GLU ASP PHE THR ARG GLY LYS LEU MET SER SER 120  
 AAA CTG GAG AAA GAA GAT TTT ACC AGG GGA AAA CTC ATG AGC AGT CTG CAC CTG AAA AGA TAT TAT GGG AGG ATT  
 450  
 120  
 LYS LEU GLU LYS GLU ASP PHE THR ARG GLY LYS LEU MET SER SER 120  
 AAA CTG GAG AAA GAA GAT TTT ACC AGG GGA AAA CTC ATG AGC AGT CTG CAC CTG AAA AGA TAT TAT GGG AGG ATT  
 500  
 130  
 LEU HIS TYR LEU LYS ALA LYS GLU TYR SER HIS CYS ALA TRP THR ILE VAL ARG VAL GLU ILE LEU ARG ASN PHE  
 CTG CAT TAC CTG AAG GCC AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC CTA AGG AAC TTT  
 550  
 140  
 LEU HIS TYR LEU LYS ALA LYS GLU TYR SER HIS CYS ALA TRP THR ILE VAL ARG VAL GLU ILE LEU ARG ASN PHE  
 CTG CAT TAC CTG AAG GCC AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC CTA AGG AAC TTT  
 600  
 160  
 TYR PHE ILE ASN ARG LEU THR GLY TYR LEU ARG ASN END  
 TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA AAC TGA AGATCTCCTAGCCTGTCCCTCTGGGACTGGACAATTGCTTCAAGCA  
 650  
 166  
 TYR PHE ILE ASN ARG LEU THR GLY TYR LEU ARG ASN END  
 TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA AAC TGA AGATCTCCTAGCCTGTCCCTCTGGGACTGGACAATTGCTTCAAGCA  
 700  
 TTCTTCAACCAGCAGATGCTGTTTAAGTGACTGATGGCTAATGTAAGTGAAGGACACTAGAAGATTTTGAATTTTATTAAATTATGAGTT  
 750  
 ATTTTATTTTATTAAATTTTATTTTGGAAAATAAATTATTTTGGTGCAAAA  
 800  
 3'

FIG. 3.



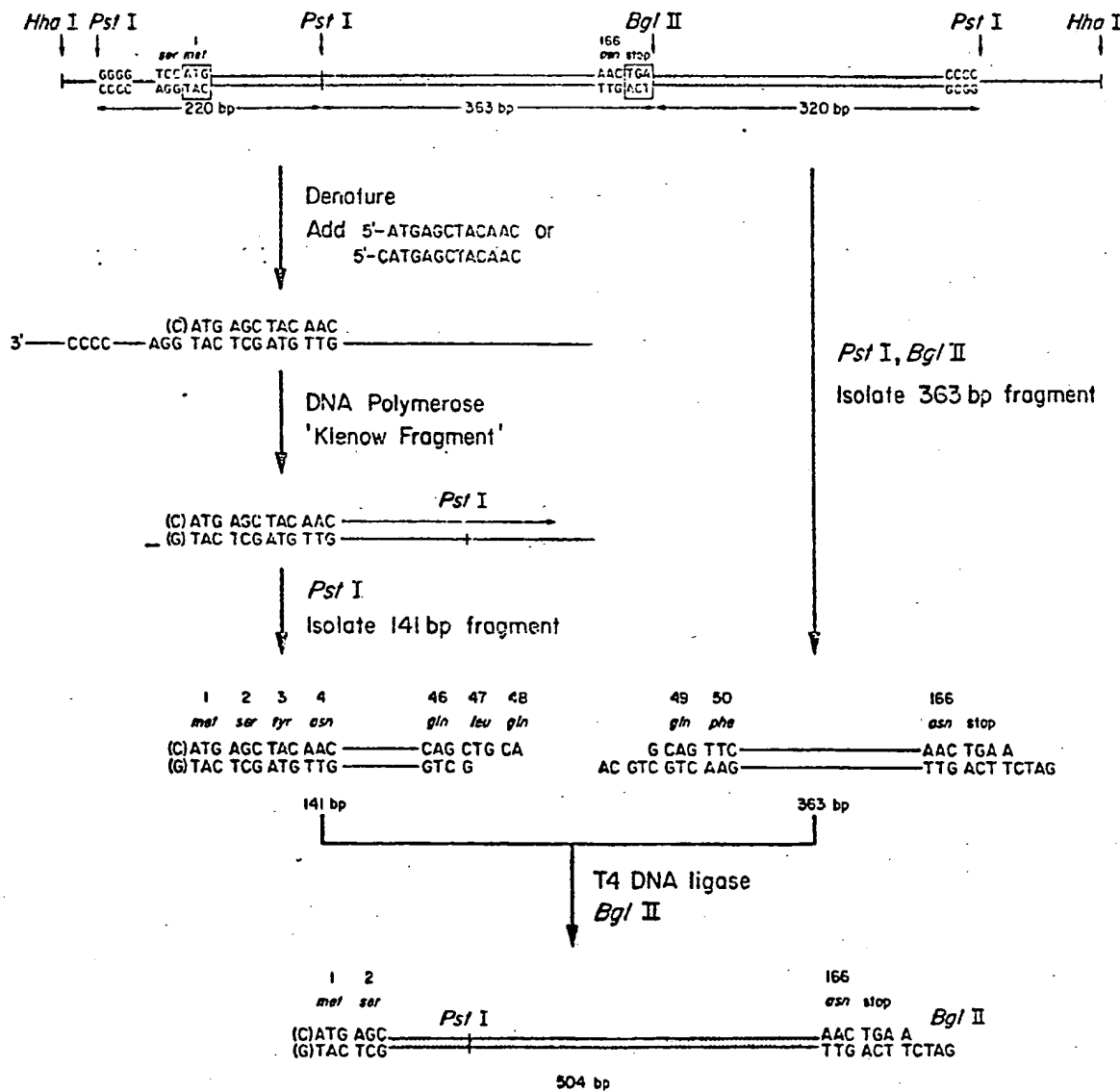


FIG. 4.

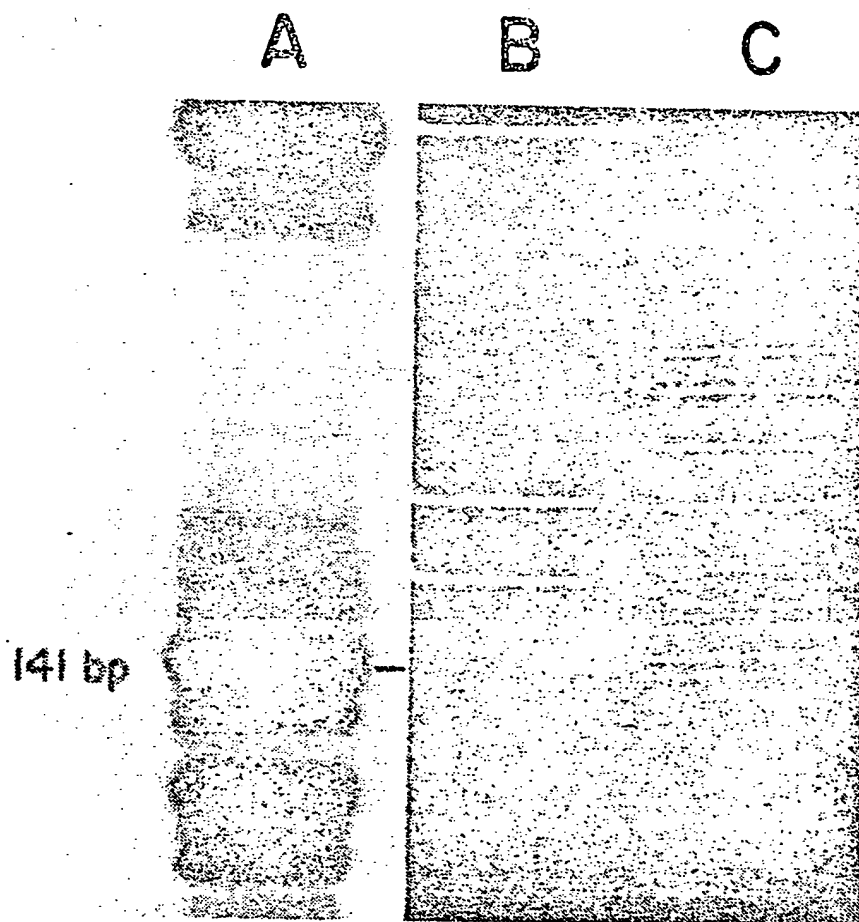


FIG. 5.

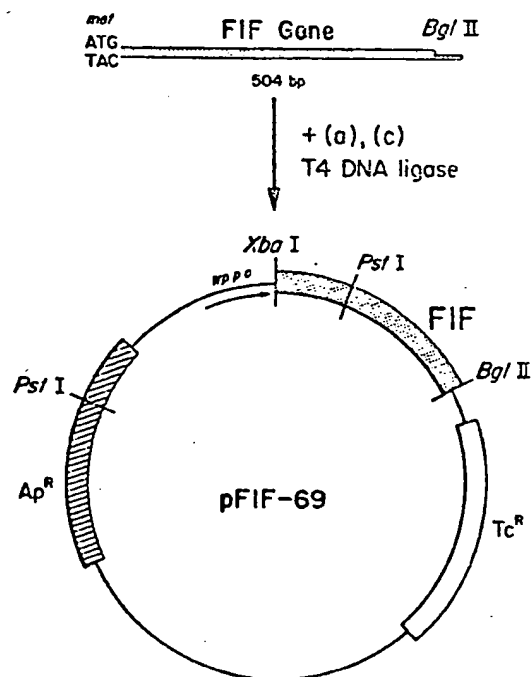
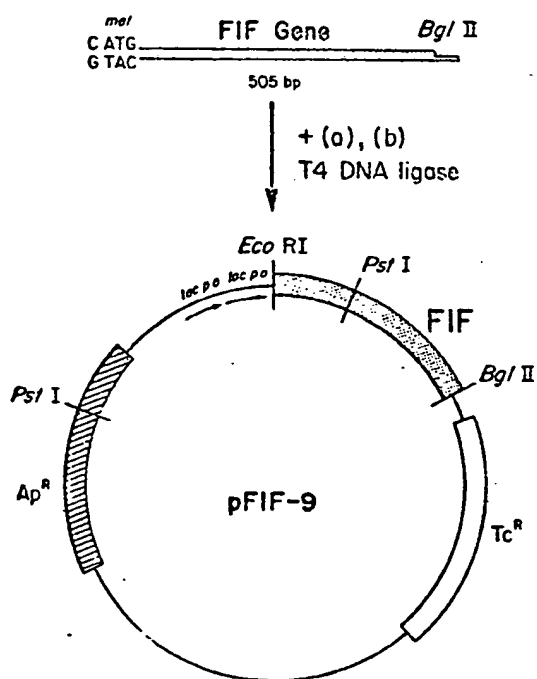
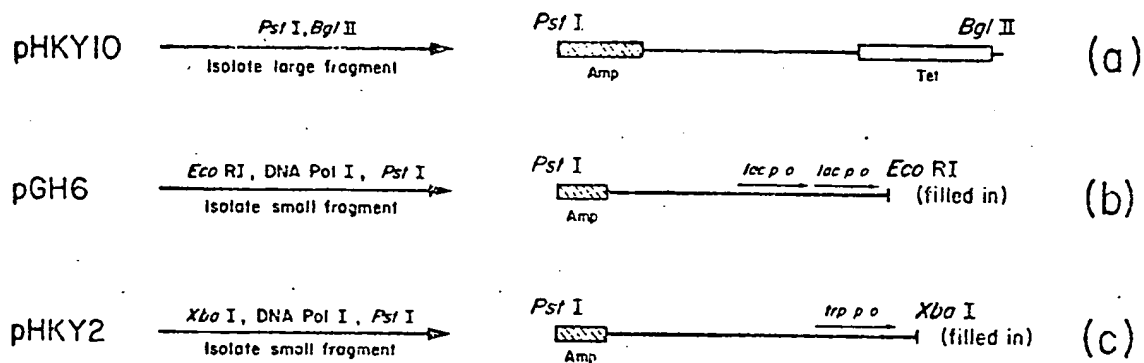


FIG. 6.

(19)



Europäisches Patentamt  
European Patent Office  
Office européen des brevets

(11) Publication number:

**0 146 413**  
**A2**

(12)

# EUROPEAN PATENT APPLICATION

(21) Application number: 84308984.8

(22) Date of filing: 20.12.84

(51) Int. Cl.<sup>4</sup>: **C 07 K 15/26, C 12 P 21/02,**  
**C 12 N 15/00, C 07 H 21/04,**  
**A 61 K 45/02**

(30) Priority: 21.12.83 GB 8334102

(43) Date of publication of application: 26.06.85  
Bulletin 85/26

(84) Designated Contracting States: DE FR GB IT

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(54) Interferons with novel cysteine pattern.

(57) Human interferons are modified by providing novel cysteine substitutions and disulfide bonds. The amino acid sequence of a first interferon is combined with the cysteine and/or disulfide pattern of a second interferon to give modified interferons with hybrid properties. DNA polymers encoding for the modified interferons are disclosed together with plasmids incorporating the DNA and the transformation of host cells with the plasmid. The modified interferons are expressed by the transformed host cells and can be formulated for pharmaceutical use.

**EP 0 146 413 A2**

TITLE: "INTERFERONS WITH NOVEL CYSTEINE PATTERN"

SPECIFICATION

Background of the Invention

1. Field of the Invention

This invention describes the use of recombinant DNA technology for the design of novel interferon molecules. These novel interferons comprise the amino acid sequence of human  
5 beta interferon modified by the addition or deletion of one or more cysteine residues thereby changing the disulfide cross-linking characteristics of the interferon.

2. Description of the Prior Art

INTRODUCTION

10 The novel feature of the invention described below is that the coding sequence of the human interferon beta gene (HuIFN- $\beta$ ) has been changed at specific points, by the process of site directed mutagenesis, to induce novel amino acid arrangements.

15 Specifically the number and arrangement of cysteine residues has been altered to produce a pattern analogous to that observed in the human interferon- $\alpha$  (HuIFN- $\alpha$ ) family. The novel arrangement is expected to impose an IFN- $\alpha$ -like tertiary structure on the IFN- $\beta$  protein sequence and hence  
20 lead to novel properties of the molecule.

The alpha and beta interferons, specifically Human IFN $\alpha_1$ ; (D) and Human IFN- $\beta$  have been shown to be structurally related. They are 45% homologous at the nucleotide level and 29% homologous at the amino acid level (Taniguchi et al., Nature 285 547 (1980). Sternberg and Cohen (Int. J. Biol. Macromol. 4 137 (1982)) have produced a model suggesting the  $\alpha$  and  $\beta$  interferons' tertiary structure may be similar. Disulfide bonds are known to influence both tertiary protein structure and stability. The tertiary structure of the Human IFN $\alpha$  has been shown to be in part dependent upon the disulfide linkages (Wetzel et al. UCLA Symp. Mol. Cell Biol. 1982, 25, 365-376)

The interferons are a class of proteins that occur in vertebrates and function as biological regulators of cell function which include increasing resistance to pathogens, limiting cell growth and modulating the immune system. The most studied property of the interferons is their ability to convert cells into an "antiviral" state during which they are more resistant to virus replication (Lengyel, Annual Review of Biochemistry, 51:251, 1982).

In addition to conferring antiviral resistance on target cells, interferons (IFNs) have both immunomodulatory and antiproliferative properties (Stewart, 1979, The Interferon System, Springer, Berlin). The IFNs, by virtue of their antigenic, biological and physico-chemical properties, can be

grouped into three classes: Type I, IFN- $\alpha$  ("leucocyte") and IFN- $\beta$  ("fibroblast"); and Type II, IFN- $\gamma$  ("immune") (Stewart et al., 1980, Nature, 286, 110). Detailed information is now available on the virus-induced, acid stable

5 IFN- $\alpha$  and IFN- $\beta$  and the mitogen-induced IFN- $\gamma$ . All three IFN cDNAs have been cloned from their respective induced mRNAs, the DNA sequenced and their potential protein sequences deduced (Taniguchi et al., 1979, Proc. Japan Acad. Ser. B 55, 461-469; Houghton et al., 1980, Nucleic Acids Res. 8, 2885-2894; Nagata et al., 1980, Nature, 284, 316-320; Nagata

10 et al., 1980, Nature, 287, 401-408; Goeddel et al., 1981, Nature, 290, 20-26; Gray et al., 1982, Nature, 295, 503-508). IFNs- $\alpha$  and IFN- $\beta$  have been purified to homogeneity and the partial protein sequences obtained confirm

15 the derived IFN- $\beta$  sequence and the sequences of some recombinant IFN- $\alpha$ 's (Allen and Fantes, 1980, Nature, 287, 408-411; Knight et al., 1980, Science, 207, 525-526; Stein et al., 1980, Proc. Natl. Acad. Sci, USA, 77, 5716-5719; Zoon et al., Science, 207, 527-528). The cysteine at the 17 position

20 of beta interferon has been replaced by serine (R. O'Connell, Genetic Technology News, 3: 2, July 1983, European Patent Application 83306221.9)

Human IFN- $\alpha$  is specified by a multigene family comprising at least 14 different genes, with at least 3 additional

25 pseudogenes and 4 other genes known to hybridize, but not yet sequenced (Weissman, 1982, 11th Annual UCLA Symposium on Molecular and Cellular Biology). In contrast, there is only

one well characterised human IFN- $\beta$  gene (Owerbach *et al.*, 1981, Proc. Natl. Acad. Sci, USA, 78, 3123-3127). The IFN- $\gamma$  gene differs from IFNs- $\alpha$  and - $\beta$  by having three introns and thus displays another distinction between the Type I and Type  
5 II IFNs (Gray and Goeddel, 1982, Nature, 298, 859-863).

Homologies exist between members of the human IFN- $\alpha$  multigene family, and between human IFN- $\alpha$  and IFN- $\beta$  genes. It appears that IFN- $\alpha$  and IFN- $\beta$  genes are the products of an ancient gene duplication, and perhaps diverged early in  
10 vertebrate evolution (Taniguchi *et al.*, 1980, Nature, 285, 547-549). In contrast, the IFN- $\alpha$  multigene family seems to have diverged much more recently, perhaps within the last 26 million years (Miyata & Hayashida, 1982, Nature, 295, 165-168).

While the mechanism of action of interferons is not completely  
15 understood, certain physiological or enzymatic activities respond to the presence of the interferons. These activities include RNA synthesis and protein synthesis. Among the enzymes induced by interferons is (2'-5')(A)<sub>n</sub> synthetase which is activated by double stranded RNA. This synthetase generates  
20 2'-5' linked oligoadenylates from ATP which activates a latent endoribonuclease, RNase L, which cleaves single stranded RNA such as messenger RNA (mRNA) and ribosomal RNA (rRNA). Interferon induces a protein kinase which phosphorylates at least one peptide chain initiation factor and inhibits protein  
25 synthesis (Lengyel, *ibid* p. 253)



Interferons have been shown to be negative growth regulators for cells by regulation of the (2'-5')A<sub>n</sub> synthetase activity (Creasey et al., Mol. and Cell Biol., 3, 780,786 1983).

IFN- $\beta$  was indirectly shown to be involved in the normal  
5 regulation of the cell cycle in the absence of inducers through the use of anti-IFN- $\beta$  antibodies. Similarly, interferons have been shown to have a role in differentiation (Dolei et al., J. Gen. Virol 46: 227-236, 1980) and in immunomodulation (Gresser, Cell. Immunol. 34: 406-415, 1977).

10 Interferons may also alter methylation patterns of mRNAs and alter the proportion of fatty acids in membrane phospholipids, thereby changing the rigidity of cellular membranes. These and other mechanisms may respond to interferon-like molecules in varying degrees depending upon the structure of the  
15 interferon-like polypeptide. It is envisaged that an IFN- $\beta$  with an IFN- $\alpha$  disulfide pattern may display a new advantageous phenotype. For example, IFNs which show a greater antiviral to antiproliferative activity (and vice-versa) or have an enhanced activity/specificity against a particular  
20 virus infected tissue or transformed cell mass.

Increased stability is an expected result of the creation of new disulfide bonds. Stability is defined as increased resistance to denaturation, proteolytic enzymes and other physical or activity changes. The increase in stability

results in improved recovery during production, increased storage life and prolonged activity in solution.

The design and synthesis of new interferon-like polypeptides composed of beta interferons with alpha interferon disulfide pattern may allow the selective activation of only part of normal interferon-induced activities. These hybrid polypeptides could then be used appropriately to activate the interferon systems. In addition, the affinity of these modified interferons for cell surface receptors may differ from that of naturally occurring interferons. This would allow selective or differential targeting of interferons for a particular cell type.

Novel interferons containing a modified cysteine pattern can be constructed for the cysteine pattern of alpha, beta or gamma interferons using the methods of this invention. That is, a human alpha interferon can incorporate the cysteine patterns of the human beta or human gamma interferons; a human beta interferon can incorporate the cysteine patterns of human alpha or human gamma interferons; and a human gamma interferon can incorporate the cysteine patterns of human alpha or beta interferons. Similarly, a combination cysteine pattern from two or more interferon classes can be substituted into the third interferon class.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a summary flow chart illustrating the amino acid changes by site directed mutagenesis resulting in IFNX802, 803 and 804.

- 5    Figure 2 illustrates the restriction map of Construction I.

Figure 3 illustrates the restriction map of Constructions II through V.

Figure 4 illustrates the site directed mutagenesis resulting in IFNX802.

- 10   Figure 5 illustrates the site directed mutagenesis resulting in IFNX803.

Figure 6 illustrates the site directed mutagenesis resulting in IFNX804.

- 15   Figure 7 illustrates the restriction map of Constructions VI, VII and VIII.

## DESCRIPTION OF THE MODIFIED INTERFERONS

Chart 1 illustrates the DNA sequence coding for the amino acid sequence of IFN802. In addition to the traditional three letter abbreviation for amino acids, at the bottom of the figure is the one letter abbreviations recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine.

Chart 2 illustrates the DNA sequence coding for the amino acid sequence of IFNX803.

Chart 3 illustrates the DNA sequence coding for the amino acid of IFNX804.

Chart 4 illustrates the DNA sequence coding for the amino acid sequence of IFNX815.

Chart 5 illustrates the DNA sequence coding for the amino acid sequence of IFNX816.

Chart 6 illustrates the DNA sequence coding for the amino acid sequence of IFNX817.

Chart 7 illustrates the DNA sequence coding for the amino acid sequence of IFNX818.

Chart 8 illustrates the DNA sequence coding for the amino acid sequence of IFNX457.

## 5 SUMMARY OF THE INVENTION

One object of this invention is the reorganization of the position and number of cysteines in HuIFN- $\beta$  to a pattern analogous to that found in the HuIFN- $\alpha$  family, by the process of site directed mutagenesis of individual nucleotides of the

10 HuIFN- $\beta$  coding sequence, so as to cause defined changes in the amino acid sequence of the HuIFN- $\beta$ . The resultant modified HuIFN- $\beta$  molecules show different or novel properties from that of HuIFN- $\beta$ , and may show properties similar to those exhibited by the HuIFN- $\alpha$  family. A summary flow chart

15 of the construction of the modified HuIFN- $\beta$  IFNX802, 803, and 804 molecules are shown in figure 1. Similarly, the disulfide pattern and amino acid sequence of the alpha, beta and gamma human interferons can be combined to form new hybrid or modified interferons. Another object of the invention is to

20 create disulfide linkages that improve the physical and pharmacological properties of modified interferons, including stability.

An object of the invention is the production of DNA sequences coding for the production of the modified-interferons IFNX 802, 803, 804, 815, 816, 817, 818 and 457 and the amino acid sequences themselves.

5 Another object of the invention is the production of a pharmaceutical composition containing a therapeutically effective amount of the modified-interferons which are useful for anti-viral, anti-proliferative, anti-tumor, immunomodulatory or immunogenic treatment. Yet another object  
10 of the invention is a method of producing the modified-interferons of the present invention utilizing microorganisms.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

##### I. Rationale for construction

15 The coding sequences of the leukocyte (alpha) interferon (IFN- $\alpha$ ) family are distantly related to the sequence of the fibroblast (beta) interferon (IFN- $\beta$ ) gene, for example the coding sequence of HuIFN- $\alpha_1$  (D) is 45% homologous at the nucleotide level and 29% homologous at the amino acid level to  
20 HuIFN- $\beta$  (Taniguchi et al, Nature 285 547 (1980)).

Secondary and tertiary structures of  $\alpha$ - and  $\beta$ -interferons have been derived by various computer modelling

procedures. Sternberg and Cohen (Int.J.Biol.Macromol. 4 137 (1982)) have produced a model for interferon tertiary structure applicable either to alpha or beta interferon, suggesting that the in vivo structures of each type of interferon are similar.

- 5 Human interferon alpha's have been shown to contain four cysteines at positions 1, 29, 98, 138 (positions relate to the IFN- $\alpha_2$  (A) sequence) which have been shown to form two intramolecular disulfide bridges with bonds between cys 1 and cys 98, between cys 29 and cys 138 (Wetzel, Nature 289 606  
10 (1981). Human interferon beta contains three cysteines, at positions 17, 31 and 141. Positions 31 and 141 in beta have been considered analogous to positions 29 and 138 in the interferon alpha family. It has been shown that alteration of cys<sup>141</sup> to tyr<sup>141</sup> abolishes interferon beta antiviral  
15 activity. Further it has been described that pre-treatment of HuIFN- $\beta$  with the reducing agent dithiothreitol abolishes antiviral activity (Shepard et al, Nature 294 563 (1981) ). These observations have been taken to show that a disulfide bridge between cys 31 --- 141 is essential for the activity of  
20 HuIFN- $\beta$ .

The cysteine substitutions in the modified interferons may be in the analogous position of another interferon or in the same sequential amino acid position. From the same sequential position, the cysteine may be moved 1 to 4 amino acid positions  
25 toward either end of the polypeptide replacing the amino acid

normally in that position. Therefore, the cysteine substituted for the third amino acid of human beta interferon may be alternatively substituted at amino acid positions 1, 2, 4, 5, 6, or 7. Similarly, the cysteine substitution at position 101, 5 may also be at positions 97, 98, 99, 100, 102, 103, 104, or 105. These alternative position substitutions result in an altered stress level on the disulfide bond formed.

## II. Outline of the technique of site directed mutagenesis

Site directed mutagenesis, using oligonucleotides to 10 produce defined sequence alterations is perhaps the most specific means of producing mutants in vitro.

The background to the method involved is reviewed in Zoller and Smith (Nucleic Acids Res. 11(20) 6487 (1982) ), which also details many of the technical aspects of the methodology.

15 To summarise the procedure, the desired nucleotide change(s) are identified and an oligonucleotide constructed which is complementary to the region 5' and 3' to the nucleotide of interest. At this position the nucleotide used is that which will be complementary to the desired mutation 20 (e.g., Figures 4-6). Thus when the oligonucleotide is annealed to the coding sequence a mismatch occurs at the point of interest.



In order to produce the coding strand in a single stranded form the most practical means is to introduce the sequence into a bacteriophage which has both double stranded and single stranded DNA phases during its life cycle. Two phages commonly  
5 used are  $\phi$ x174 and M13. The bacteriophage M13 was used in this method.

After annealing of the oligonucleotide to the sequence to be mutated, closed circular molecules are transcribed and ligated. The complete molecules may be separated from  
10 incomplete transcripts either by separation on alkaline sucrose gradients (Zoller and Smith, 1982) or by electrophoresis and elution from low gelling temperature agarose. The latter technique has the advantage that subsequent transformation of the DNA into competent E.coli JM101 cells can be done in the  
15 presence of the agarose, as will be described, so reducing the number of manipulations.

Prior to separation of transcripts, by whichever means, incomplete transcripts may be degraded by  $S_1$  nuclease to allow greater resolution during the separation stage. After  
20 transformation a population of mutant and wild type molecules are obtained which may be distinguished by one of a number of screening techniques.

For example, the desired change may introduce or delete a restriction endonuclease site which can be easily detected.

Alternatively, the difference in  $T_m$  (point of 50% irreversible melting) of hybrids formed between the oligonucleotide primer and either the original sequence of the mutant sequence can form the basis of a hybridization screening  
5 procedure (e.g., Zoller and Smith, 1982).

In order to increase the yield of mutants, the pool of transformed cells can be used to prepare a mixed population of single stranded (ss) DNA molecules. These can then be used as a template for a further round of annealing/transcription under  
10 conditions favouring the annealing of the oligonucleotide to the mutant sequence rather than the original sequence.

The DNA sequences illustrated in Charts 1 to 8 illustrate one preferred example of a DNA sequence that will code for the synthesis of the modified interferon amino acid sequence  
15 illustrated on the same chart. The genetic code (Lehninger, Biochemistry, Worth Publishing p. 962) will allow the design and synthesis (Nucleic Acids Research 11 477, 1983) of additional DNA sequences which also code for the amino acid sequences described in Charts 1 to 8. Such additional  
20 sequences could also be utilized in the plasmids pJA1, pJA2 and pJA3 to produce the modified-interferons of the present invention.

1. ConstructionsTABLE 1

## NOMENCLATURE FOR CONSTRUCTIONS

5	<u>CONSTRUCTION</u>	<u>TRIVIAL NAME</u>	<u>PRODUCT</u>
			<u>IDENTIFICATION</u>
	I	M13-1RB-00	HuIFN- $\beta$
	II	M13-4AB-00	HuIFN- $\beta$
	III	mJA1	HuIFN-X802
	IV	mJA2	HuIFN-X803
10	V	mJA3	HuIFN-X804
	VI	pJA1	HuIFN-X802
	VII	pJA2	HuIFN-X803
	VIII	pJA3	HuIFN-X804

M13 is a bacteriophage, a bacterial virus, more  
 particularly, a so-called "filamentous phage", which infects  
 male Escherichia coli (E.coli) cells. Unlike many  
 bacteriophages, the DNA found in the virus or phage particle is  
 single stranded (ss DNA). Upon infection of an E.coli cell,  
 the ss DNA is converted to the corresponding double stranded  
 form (ds DNA) and amplified, under suitable conditions of  
 growth, to, for example, from 200 to 300 copies per cell. The  
 phage-infected cells do not lyse releasing the new phage  
 particles, but continue to grow and divide, although at a  
 reduced rate. This may be seen on agar plates as a lighter

area, a "plaque", of slower-growing cells against a background of uninfected cells.

The ds DNA is also termed the "replicative form" (RF). This is comparable to a bacterial plasmid and may be used as a cloning and expression vector. The RF is central to the phage multiplication process. It is the template for ss DNA synthesis and for expression via protein biosynthesis of M13 phage genetic information. The former process produces phage particles and the latter allows the synthesis of phage proteins which are required to direct infected cells to produce further phage particles.

Wild type M13 has previously been modified for use as a cloning vehicle by the insertion of a fragment of E.coli DNA containing the lactose operon control region (lac promoter) and coding information for an active  $\beta$ -galactosidase ( $\beta$ -gal) in a non-essential region of the phage DNA. When the lac promoter is active, the expression of  $\beta$ -gal occurs and this is detected by a simple blue colour reaction in the infected cells. However, the cloning of DNA fragments generally results in the interruption of the lac Z gene and hence in colourless plaques due to the failure of  $\beta$ -gal expression. Thus, recombinants may be detected visually.

For example, U.K. Patent No. 1,588,572, which is directed to the production of filamentous hybrid phages, is representative of the stage of the art outlined above.

The known system allows the cloning of different-sized fragments of foreign DNA and the identification of recombinant clones without relying on the use of antibiotic resistance genes. Also, it allows the possibility of easily purifying  
5 phage ss DNA for use in recombinant characterisation and DNA sequencing, and site directed mutagenesis.

The known bacteriophage M13 mp 701 may be regarded as a starting point for the present invention. This vector, which is freely available, was constructed from M13mp7, which is  
10 commercially available, by known methods. Similarly, M13mp7 was in turn constructed from M13mp2. Reference may be made in this connection to, for example, Messing, J., et al., Proc.Natl.Acad.Sci.U.S.A., 74, 3642. (1977).

The plasmids pJA1, pJA2 and pJA3 in E coli K12 strain  
15 HB101 have the following ATCC designations; 39520, 39521 and 39522.

The American Type Culture Collection (ATCC) is located at 12301 Parklawn Drive, Rockville, MD 20852 USA.

#### Example 1

20 Construction I (M13-IRB-00, HuIFN-8)

Recloning of a human fibroblast interferon gene plus trp promoter to give M13-1RB-00 (see Fig. 2):

A 1172 bp DNA fragment containing the trp promoter followed by the mature HuIFN- $\beta_1$  gene bounded on the left by an

5 EcoRI site and on the right by a Bam HI site (GB Patent Application No. 2,068,970) was recloned between the EcoRI and Bam HI sites of phage M13 mp 701 as follows:

The joining of the EcoRI-Bam HI fragment containing the HuIFN- $\beta_1$  gene to the EcoRI-Bam HI digested M13 mp 701

10 vector was performed in an incubation of 50  $\mu$ l containing: 0.25  $\mu$ g vector; 0.9  $\mu$ g EcoRI-Bam HI cut p1/24 (GB P). Approximately 8% of plaques on each plate were colourless, indicating the presence of recombinant phages (1RB-00). Recombinants were firmly identified by size and by nucleotide  
15 sequence analysis, also by expression of antiviral activity.

To prepare sufficient ss DNA for nucleotide sequence analysis, colourless plaques were picked and added to 2.5 ml YT medium containing 25  $\mu$ l of a dense, overnight culture of E.coli K12 JM101. Phage was grown by aeration for 5 hours at  
20 37°C and the ss DNA purified by known methods, (see, for example, Sanger, F., et al, (J. Mol. Biol., 143, 161, 1980). The ss DNA was used as the template for dideoxy sequencing, (see, for example, Sanger, F., et al, Proc. Natl. Acad. Sci. U.S.A., 74, 5463, 1977). For example, the presence and

sequence of the trp promoter and the presence of the HuIFN- $\beta$ 1 gene was established with an oligonucleotide primer, IFIA (GB Patent Application No. 2,068,970) which is known to prime in the HuIFN- $\beta$  coding region.

5    Example 2

Construction II. (M13-4AB-00, HuIFN- $\beta$ )

Deletion of the lac promoter from M13-1RB-00(I) to give a recombinant capable of expressing mature HuIFN- $\beta$  under the control of only the trp promoter, II = M13-4AB-00) (See Fig.  
10    3).

Construction of this clone was achieved by excision of a 406 bp Ava I-EcoRI fragment as follows: Ava I-EcoRI double digestion was effected in 100  $\mu$ l containing: 10  $\mu$ g ds DNA prepared from construction I, (see, for example, Birnboim,  
15    H.C., and Doly, J., Nucl. Acids Res., 7, 1513, 1979), 6 mM Tris-HCl, pH 7.5, 10 mM  $MgCl_2$ , 6 mM 2-mercaptoethanol, 30 mM NaCl, 15 units Ava I and 15 units EcoRI for 90 minutes at 37°C. The DNA was precipitated by the addition of 20  $\mu$ g tRNA; 0.3 M NaAc, pH 4.5 to 0.3 M final concentration and 0.3  
20    ml ethanol for 10 minutes at -70°C. Repair of protruding 5'-ends, with DNA polymerase, was then done in a 50  $\mu$ l final volume.

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To "fill-in" protruding 5'-ends, the DNA fragments were repaired in vitro with DNA polymerase I (Klenow fragment) in a 50  $\mu$ l reaction containing 1 $\mu$ g DNA in 10 mM NaCl, 50 mM Tris-HCl, pH 7.8, 10 mM  $MgCl_2$ , 1 mM 2-mercaptoethanol, 0.2 mM  
5 each deoxynucleoside 5'-triphosphate, 20  $\mu$ g/ml. bovine serum albumin, 0.2 mM each deoxynucleoside 5'-triphosphate, and 1 unit of Klenow enzyme for 20 minutes at 14°C in a volume of 50  $\mu$ l, then for 10 minutes at 65°C.

The repaired DNA was self-ligated in a 50 $\mu$ l incubation  
10 containing 5  $\mu$ l of the above incubation (equivalent to 0.1  $\mu$ g DNA), 66 mM Tris-HCl, pH 7.5, 5 mM  $MgCl_2$ , 20mM DTT, 1mM ATP, and 4.5 units T4 DNA ligase for 17 hours at 25°C. Uptake of DNA into  $CaCl_2$ -treated E.coli K12 JM101 and plating out of cells for plaques was performed using standard methods.

15 The identity of the new clone (M13-4AB-00) was confirmed by Tag I restriction analysis.

### Example 3

#### Construction III (mJA1), HuIFN-X802)

Alteration of Human Interferon $\beta$  (HuIFN $\beta$ ) amino acid 3  
20 (tyrosine) to cysteine, to give mJA1. (See Figs. 3 and 4 and Chart 1)



- 21 -

A tetradecamer of the sequence 5' OH-AGT TGC AGC TCA TG-OH (Fig. 4) was constructed using standard phosphotriester chemistry; Reference: Nucleic Acids Research 11 477 (1983). This sequence is complementary to the sequence 5'C ATG AGC TAC AAC T which consists of the nucleotide preceding the initiator ATG followed by the first 13 nucleotides of the coding sequence of Human IFN- $\beta$  - (GB Patent Application No. 2,068,970) with the exception that the 6th nucleotide of the primer, numbered from the 5' end corresponds to the complement of the desired mutation. This mutation will change the nucleotide A at position 8 of the coding sequence to a G.

Specific priming of the synthetic oligonucleotide to the region which was to be mutated was observed by the following procedure. First the oligonucleotide was labelled with [ $^{32}\text{P}$ ] at the 5' terminus. 10 pmole of oligonucleotide was incubated in a total volume of 50  $\mu\text{l}$  consisting of 50 mM Tris-HCl pH 7.6, 10 mM  $\text{MgCl}_2$ , 0.1 mM EDTA (ethylene diamine tetra acetic acid), 10 mM dithiothreitol, 0.1 Mm spermidine, 50  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ] ATP (5000 Ci/mmole, Amersham) and 5 units of polynucleotide kinase. The reaction mix was incubated at 37°C for 60 minutes, followed by 65°C for 5 minutes. The oligonucleotide was separated from unincorporated [ $\gamma$ - $^{32}\text{P}$ ] ATP by differential elution from a column of Whatman DE52 ion exchange resin (0.3 ml bed volume). The oligonucleotide was eluted in 0.5 M NaCl, 10 mM Tris HCl pH 7.5, 1 mM EDTA, 20  $\mu\text{g}$  E.coli tRNA was added and the oligonucleotide was precipitated

at -70°C after the addition of three volumes of ethanol. The oligonucleotide was redissolved in 8 µl of 10 mM Tris HCl pH 7.5, 1 mM EDTA.

5 pmoles of [<sup>32</sup>P] oligonucleotide were annealed to 0.5  
5 pmole of M13-4AB-00 in a total volume of 5 µl of 10 mM NaCl, 40 mM Tris-HCl pH 7.5, 20 mM MgCl<sub>2</sub>, 2 mM 2-mercaptoethanol, by heating at 80°C for 5 minutes followed by incubation at 20°C for 1 hour.

The samples were adjusted to 67.5 mM NaCl, 45 mM Tris-HCL  
10 pH 7.5, 25 mM MgCl<sub>2</sub>, 1 mM 2-mercaptoethanol, 83 uM each of dATP, dCTP, dGTP, dTTP and 0.5 unit of the 'Klenow' fragment of DNA Polymerase I (BRL Inc.) in a final volume of 15 µl. The samples were incubated at 25°C for 4 hours. The samples were diluted eight fold with 10 mM Tris-HCL pH 7.5, 1 mM EDTA,  
15 heated to 65°C for 5 minutes, adjusted to 0.3 M Na acetate pH 4.6 and finally the nucleic acid was precipitated with three volumes of ethanol at -70°C for 15 minutes. In order to identify specific priming products the samples were digested with the enzyme EcoRI and the products separated by  
20 electrophoresis through a 7M Urea, 6% Acrylamide, 0.3% Bis (NN'-methylene bisacrylamide) gel prepared in 135 mM Tris-HCL pH 8.8, 45 mM Na borate, 1 mM EDTA.

A specific transcription product of 150 bases was identified which corresponds to the instance between the

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upstream E.coli site in 4AB-00 and the 5' end of the oligonucleotide, given specific priming.

Formation of complete closed circular molecules was achieved as follows: The oligonucleotide was phosphorylated as described earlier except that ATP at 1mM final concentration  
5 replaced the [ $\gamma$ - $^{32}$ P] ATP. The oligonucleotide was annealed as described earlier except that a primer to template ratio of 100 was used (50 pmole oligonucleotide per 0.5 pmole template).

The transcription and ligation of the product was performed  
10 as follows: 10 $\mu$ l of annealed primer-template, containing 0.5 pmole of template and 50 pmole of primer in 100 mM NaCl, 40mM tris-HCl pH 7.5, 20mM MgCl<sub>2</sub>, 2mM 2-mercaptoethanol was adjusted to a volume of 20 $\mu$ l containing 20 $\mu$ Ci [ $\alpha$ - $^{32}$ P] dATP (2000ci/mmmole) 250 $\mu$ M each of dATP, dCTP, dGTP, dTTP, 0.8  
15 unit of Klenow DNA polymerase I, 2 units T<sub>4</sub> ligase (BRL Inc.) in 67.5mM NaCl, 45mM Tris pH 7.5, 25mM MgCl<sub>2</sub>, 1mM 2-mercaptoethanol. After 30 minutes at 22°C dATP was added to 250 $\mu$ M and a further 0.8 unit of Klenow DNA polymerase was added. The mixture was incubated a further 2.5 hours at 22°C.  
20 The DNA was adjusted to 0.3M Na acetate pH 4.6, and precipitated with three volumes of ethanol at -20°C overnight (16 hrs).

Incomplete transcripts were digested with S1 nuclease as follows. The DNA was incubated for 30 minutes at 25°C in a

solution of 300mM NaCl, 5mM ZnCl<sub>2</sub>, 30mM Na acetate pH 4.5 at an initial template concentration of 1nM in a final volume of 250µl. S1 nuclease was added at the rate of 1 unit per 0.01 pmole of initial template.

5        The products were added directly to 0.4 mls of competent E.coli JM101 at 0°C. After 40 minutes the cells were heat shocked for 2 minutes at 42°C then diluted into 20 ml of YT broth (8g tryptone, 5g yeast extract, 5g NaCl per litre) at 37°C. The cells were grown at 37°C for 16 hours. The cells  
10       were pelleted by centrifugation and phage in the supernatant were stored in 60% glycerol at -20°C.

Dilutions of phage were plated on indicator cells and plates containing 2-400 plaques were used to make  
nitrocellulose replicates for hybridization, using the [<sup>32</sup>P]  
15       phosphorylated oligonucleotide as probe in a manner similar to that of Benton and Davis (Science 196, 180, 1977). Filters were prewashed in 6 x SSC at 40°C for 3 hrs and hybridized using 300µl of a solution of 6 x SSC/10 x Denhardts/0.1% SDS containing primer at 400pM for 16 hrs at 40°C, under paraffin  
20       oil [1 x SSC = 0.15M. NaCl; 0.015M Na citrate, pH 7.2. 10 x Denhardts = 0.2% Bovine Serum Albumin (BSA); 0.2% polyvinyl pyrrolidone (PVP); 0.2% Ficoll]. Filters were washed in 6 x SSC/0.1% SDS at 15°C, with six changes of five minutes each.

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Fifty plaques which showed hybridization above background were picked into 50µl each of LTB (10mM Tris-HCl pH 7.5, 20mM NaCl, 1mM EDTA). Cellular debris was spun out and 1µl of supernatant for each plaque was spotted onto a lawn of E.coli JM101 which had been allowed to grow for 60 minutes at 37°C. After overnight growth large plaques were formed on the lawn of E.coli JM101. Nitrocellulose replicates were again taken and hybridised as above. Nineteen of the 50 plaques showed hybridization above control levels. These were rescreened exactly as described above. Six were taken for further analysis by DNA sequencing. As the desired change was A to G, the coding strand was analysed by 5'end-labelling followed by the G reactions of the Maxam and Gilbert chemical degradation sequencing technique (Maxam and Gilbert, Methods in Enzymology Vol 65 (1), p 499, 1980) while the non-coding strand was analysed by 3'end-labelling followed by the 'C' reaction of the Maxam and Gilbert technique. Thus mutants would exhibit an extra G in the coding strand and an extra C in the non-coding strand compared to parallel reactions on the parent M13-4AB-00. The DNAs were therefore digested with either Hind III to produce staggered ends for 3' labelling, or Hpa I to produce a blunt end for 5' labelling.

After labelling the fragments were digested with Bgl II to separate the labelled ends. The required fragments were isolated by electrophoresis on a 10% Acrylamide/0.33% bis-acrylamide gel in 135mM Tris-HCl pH 8.8, 45mM Na borate,

1mM EDTA. The fragments were electroeluted and concentrated on a 0.3ml Whatman DE52 ion exchange resin column prior to DNA sequencing. All six clones showed the desired change. Two of the clones were picked and tested to show that the gene product  
5 was still antivirally active.

Interferon anti-viral assays were performed on extracts of M13 infected or plasmid transformed cells as follows: Fifty 200ml. cultures in tryptophan-free minimal medium plus glucose were harvested at an optical density (600 nm) of 0.6-0.9 by  
10 centrifugation at 10,000 rpm for 10 minutes. The cells were then frozen at -70°C, thawed in the presence of 2.5-5.0ml of 15% (w/v) sucrose; 50 mM Tris-HCl, pH 8.0; 0.1% (w/v) human serum albumin and 2.5 mg lysozyme, then incubated at 20°C for 15 minutes with shaking. The cell debris was removed by  
15 centrifugation at 15,000 rpm for 20 minutes and the supernatant was further clarified and sterilised by filtration through a 0.22 um pore diameter nitrocellulose filter. Finally the extract was assayed for anti-viral activity by monitoring the protection conferred on Vero (African green monkey) cells  
20 against the cpe (cytopathic effect) of EMC (Encephalomyocarditis) virus infection in an in vitro microplate assay system (see, for example, Dahl, H., and Degre, M., Acta. Path. Microbiol. Scan., 1380, 863 1972).

Similar levels of antiviral activity to that shown by the  
25 parent were observed. Single-stranded phage DNA was isolated

from one clone by established procedures, to act as template for construction IV.

Example 4

Construction IV (mJA2, HuIFN-X803)

5       Alteration of mutant interferon  $\beta$  (mJA1) at amino acid 101 (valine) to cysteine, to give mJA2. (See Figures 3- and 5 and Chart 2).

10       An octadecamer of sequence 5' OH-CTTCCAGGCATGTCTTCA-OH 3' (Fig. 5) was constructed using phosphotriester chemistry, as for Construction III. This sequence is complementary to the sequence 5'TGAAGACAGTCCTGGAAG.3' which comprises nucleotides 294 to 310 of the IFN- $\beta_1$  coding sequence (see published GB Patent Application No. 2,068,970) with the exception that the 9th and 10th nucleotides of the oligonucleotide, numbered from 15 the 5'end, correspond to the complement of the desired mutations. Thus the desired changes will be from G at nucleotide 301 of the coding sequence to T, and from T at nucleotide 302 to G.

20       Specific priming was demonstrated exactly as for mJA1. The specific product was identified as a 348 base fragment after digestion of the transcription products with Hpa I and electrophoresis as previously described.

Formation of closed circular molecules, ligation, transfection were as described from Example 3. Phage DNA was prepared from the total pool of transformants and used as a template for reannealing of the primer, transcription, ligation,  $S_1$  nuclease treatment, and transfection as described, except that in the  $S_1$  reaction the conditions were changed to encourage digestion at the mismatch between parent template and primer, so enriching for mutant closed circular molecules in the population. The reaction therefore was performed in 300  $\mu$ l of 100 mM NaCl, 30mM Na acetate Ph 4.5, 5mM ZnCl and containing 0.8 pmole of initial template and 2 units of  $S_1$  nuclease.

Phage DNA was prepared from the total pool of transformants and the enrichment stage was repeated again exactly as above. 50 plaques from the second round of enrichment were grown in 1ml (YT medium). 2 $\mu$ l of each suspension was spotted directly onto nitrocellulose and hybridised as above. Three positive plaques and two negative plaques were rescreened. Phage DNA was prepared and concentrated by polyethylene glycol (PEG) precipitation. Phage were resuspended in 50 $\mu$ l of LTB, representing a 300 fold concentration. 2 $\mu$ l of each suspension was spotted onto nitrocellulose and hybridised with [ $^{32}$ P] phosphorylated primer as described above. Hybridization confirmed the three positive plaques. The presence of the desired change was confirmed by DNA sequencing (Maxam and Gilbert). Single stranded phage DNA was prepared by



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established procedures. A short oligomer complementary to the sequence coding for amino acids 114 to 117 of the  $\beta$  sequence was prepared and phosphorylated using [ $\gamma$ - $^{32}$ P] ATP as described previously. This was annealed to the phage DNA as described and short transcripts produced which were cleaved with Pst 1. A band of 213 b. corresponding to the specific priming product was isolated on a 7M urea, 8% Acrylamide 0.2% Bis-acrylamide 135mM Tris-HCl pH 8.8, 45mM Na borate, 1mM EDTA gel. The fragment was electroeluted and the DNA sequenced using the Maxam and Gilbert technique. The DNA sequence confirmed the desired changes (Fig. 5, 6). One clone was picked and tested to show that the gene product was still antivirally active as described earlier. This clone was used as a template for construction V.

15 Example 5

Construction V (mJA3, HuIFN-X804)

Alteration of mutant human interferon  $\beta$  (mJA2) at amino acid 17 (cysteine) to serine to give mJA3 (See Fig. 3 and 6 and Chart 3).

20 A hexadecamer of sequence 5'OH-CTGACTCTGAAAATTG3' (See Fig. 6) was constructed using phosphotriester chemistry, as for Construction III and IV. This sequence is complementary to the sequence 5'CAATTTTCAGTGTGAG3' which comprises nucleotides 39 to 54 inclusive of the IFN- $\beta$  coding sequence (see published GB

Patent Application No. 2,068,970) with the exception that the 6th nucleotide of the oligonucleotide, numbered from the 5'end,, corresponds to the complement of the desired mutation. Thus the desired change will be from T to A at nucleotide 49 of the coding sequence.

Specific priming was demonstrated as for Example 3 and 4. The specific product was identified as a 187 base fragment after digestion of the transcription products with EcoRI and electrophoresis as previously described.

Formation of closed circular molecules and ligation was as described for constructions III and IV. Closed circular molecules were separated from incomplete products by electrophoresis through 1% low melting temperature agarose in 67.5 mM Tris-HCl pH8.8, 22.5mM Na borate, 0.5mM EDTA, 1µg/ml ethidium bromide without prior  $S_1$  nuclease treatment. The region corresponding to closed circular double stranded full length molecules was visualised under long wave (366nm) transillumination, cut from the gel and melted at 60°C for 5 minutes. A volume corresponding to 0.3 pmole of double stranded product was used to transfect E.coli JM101 by established procedures. The transfected cells were plated out in top agar directly. After overnight growth, nitrocellulose replicates were taken and hybridized with [ $^{32}$ P] phosphorylated primer as described. The hybridization temperature was 42°C for 36 hours. E.coli DNA (10 µg/ml,

heat denatured) was included in the prehybridization solution. Finally, the filters were washed at 44°C in 6 x SSC.

The 24 plaques which showed a signal above background were picked and grown for 6 hours in 1ml YT broth containing 25µl of log phase E.coli JM101. The cells were removed by centrifugation and the phage in solution were concentrated 80 fold by PEG precipitation, to a final volume of 10µl. 4µl of each was spotted onto nitrocellulose and hybridized using the [<sup>32</sup>P] primer. About 50% of the spots showed a signal greater than the background level. Four of the positive phage were further plaque purified. Phage was isolated from distinct, positively hybridizing plaques and the dsDNA replicative form (RF) prepared. The presence of the desired change was inferred by the appearance of a novel Hinf I site. The desired change, T to A, introduces the sequence 5' GAGTC which is a recognition sequence from the enzyme Hinf I. The presence of the site causes a Hinf I fragment of 197 base pairs in the parent RF to be cleaved to two molecules of 169 and 28 base pairs in the mutant RF.

Thus 5µg of RF was digested with 12.5 units of Hinf I in a total volume of 500µl of 6mM Tris-HCl pH 7.5, 6mM MgCl<sub>2</sub>, 6mM 2-mercaptoethanol, 50mM NaCl. for 16 hours at 37°C. The fragments were labelled at their 3'ends with [<sup>32</sup>αP] dATP as the restriction enzyme cleaves between the G and A of the recognition site. Thus the reaction consisted of 0.4 pmole of

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DNA, 20 $\mu$ Ci [ $\alpha$ -<sup>32</sup>P] dATP (2000Ci/mmol) and 2 units of Klenow DNA polymerase I in 50 $\mu$ l of 6mM Tris-HCl pH 7.5, 50mM NaCl, 6mM MgCl<sub>2</sub>, 7mM mercaptoethanol, at 25°C for 60 minutes. The fragments were separated on 10% acrylamide, 0.33% Bis-acrylamide, in 135mM Tris-HCl pH 8.8, 45mM Na borate, 1mM EDTA. All of four plaques analysed showed the desired restriction pattern indicating the desired mutation had been induced.

#### Example 6

#### 10 Constructions VI, VII, VIII

VI: pJA1, HuIFN-X802

VII: pJA2, HuIFN-X803

VIII: pJA3, HuIFN-X804

15 Subcloning of mutant  $\beta$  constructs into pMN39-1. (See Fig. 7).

Plasmid pMN39-1 consists of a deletion of 434 bp between the Bgl II and Bam HI site of plasmid p1-24. pMN39-1 therefore contains the natural HuIFN- $\beta$  gene under trp attenuator minus control. The trp control region and 161 amino acids of the IFN- $\beta$  gene are present on a 621 bp EcoRI/BstEII fragment. This fragment can be removed and replaced by the analogous fragments from mJA1, mJA2 or mJA3 to produce pJA1, pJA2, pJA3 respectively. These constructs would

thus represent the mutant HuIFN- $\beta$  genes under trp control on a high copy number plasmid also coding for the  $\beta$ -lactamase gene so allowing selection by conferring ampicillin resistance on a transformed E.coli cell.

5        In order to achieve the subcloning RF from mJA1, mJA2, mJA3, and closed circular plasmid pMN39-1 were digested with the enzymes EcoRI and BstEII. One pmole each of mJA1, mJA2 and mJA3 were digested with 10 units of BstEII for 16 hours at 37°C in a total volume for each reaction of 250  $\mu$ l. 2 pmole  
10      of pMN39-1 was digested with 10 units of BstEII for 16 hours at 37°C in a total volume of 250  $\mu$ l.

         The DNAs were precipitated and redigested with 20 units each of EcoRI for 16 hours at 37°C in a total volume of 250  $\mu$ l. The digestion products were precipitated and redissolved  
15      in 20  $\mu$ l of 10 mM Tris-HCl pH 7.5, 1 mM EDTA. The products of the digestion of pMN39-1 were resolved on a 0.8% low melting temperature agarose gel in 67.5 mM Tris-HCl pH 8.8, 22.5 mM Na borate, 0.5 mM EDTA containing 1  $\mu$ g/ml ethidium bromide. The 3303 bp EcoRI/BstEII fragment was cut from the gel and melted  
20      at 60°C.

         The products of the digestion of mJA1, mJA2, mJA3 were resolved as above except that the agarose concentration was 2%. The 621 bp EcoRI/BstEII fragment from each digest was cut from the gel and melted at 60°C.

- 34 -

A volume calculated to contain 0.17 pmole of the 3.3 kb pMN 39-1 fragment was mixed with a volume calculated to contain 0.4 pmole of the 0.62 kb fragment for each of mJA1, mJA2, mJA3. The fragments were ligated with 1 unit of T4 ligase in a total  
5 volume of 200  $\mu$ l of 65 mM Tris-HCl pH 7.5, 5mM Mg Cl<sub>2</sub>, 20 mM dithiothreitol, 1 mM ATP for 24 hours at 20°C.

Fifty  $\mu$ l of each ligation mix was used to transform 0.3 ml of competent E.coli K12 HB101 cells by established procedures.

10 Several transformants for each ligation were grown and plasmid DNA prepared by the method of Birnboim and Doly (Nucleic Acids Res. 7 1573, 1979). The plasmid DNA was digested with HinfI and compared against pMN39-1 as described for the analysis of Construction V. For pJA1 and pJA2 the  
15 digestion pattern was shown to be identical to pMN39-1. The HinfI digest of pJA3 differed from that of pMN39-1 in that the 197 bp band was not present but was replaced by bands of 169 and 28 bp, as expected.

Example 7Antiviral properties of modified IFN- $\beta$ 

Antiviral assays were performed as described in the description of the construction of mJA1 (Construction III).

5 In the cases of constructions VI - VIII the medium contained ampicillin at 100 ug/ml.

Yields of interferon were obtained for constructions I-IV as indicators that the constructs still retained biological activity (Table 2). The A600 was 0.4 when induced and 1.0 when  
10 harvested. In all cases expression from the trp promoter could be detected in inducing conditions. However, yields ranged from  $6 \times 10^3$  to  $5.3 \times 10^4$  IU./L for mJA3 (Construction II),  $3.7 \times 10^3$  to  $7.9 \times 10^4$  IU/L for mJA1 (Construction III), and  $1.45 \times 10^3$  to  $2 \times 10^5$  IU/L for mJA2 (Construction IV).

15

TABLE 2

<u>Experiment No.</u>	<u>Construct (Trivial Name)</u>	<u>Antiviral Titre</u>	
		<u>IU/L</u>	<u>Mean IU/L</u>
1.	PMN39-1	$2.3 \times 10^7$	$2.3 \times 10^7$
	VI pJA1	$4.8 \times 10^5$	$6.2 \times 10^5$
	VI pJA1	$7.6 \times 10^5$	
20	VII pJA2	$1.14 \times 10^6$	$1.2 \times 10^6$
	VII pJA2	$1.33 \times 10^6$	

- 36 -

2.		PMN39-1	$3.5 \cdot 10^7$
	VII	pJA2	$6.65 \times 10^6$
3.		PMN39-1	$6.35 \times 10^6$
	VII	pJA2	$3 \times 10^5$
5	VIII	pJA3	$2.1 \times 10^6$

The antiviral titres of the plasmid constructs VI to VIII have been investigated. Experiments (Table 2) indicated a reduction in the titre compared to the parental plasmid PMN39-1. In particular the A to G alteration from PMN39-1 to pJA1 results in an apparent drop in the titre of more than one order of magnitude.

#### SUMMARY OF TABLE 2

<u>Construct (Trivial Name)</u>	<u>Antiviral Titre (IU/L)</u>
---------------------------------	-------------------------------

	PMN39-1	$2.14 \times 10^7$
15	pJA1	$6.2 \times 10^5$
	pJA2	$2.35 \times 10^6$
	pJA3	$2.1 \times 10^6$

Data from heterologous cell assays performed with crude lysate material indicate no alteration in activity between the PMN39-1 product and that of pJA1 and pJA2.



Determination of -SH groups in proteins

The presence of sulfhydryl groups S in the modified interferons is determined by spectrophotometric titration with p-mercuribenzoate by the method of Boyer (1954). Boyer P.D. 5 (1954), J.Amer.Chem. Soc. 76,4331. This method is performed in, for example, 8M urea or 0.5 to 1% sodium dodecyl sulphate to "unmask" buried -SH groups. Other methods based on alkylation reactions are appropriate, e.g. use of N-ethylmaleimide (NEM). Gregory, J.D. (1955), J.Amer.Chem.Soc. 10 77,3922. One can also use methods based on reactions with disulphides e.g. 5,5'-dithiobis (2-nitrobenzoate) by the method of Ellman (1959). Arch.Biochem. and Biophys. 82,70, yielding a chromophore which may be quantitated spectrophotometrically.

Determination of -S-S- groups in proteins

15 The methods used for determining the sulfhydryl (-SH) content of proteins are applicable to the determination of the (-S-S-) disulfide (-S-S-) content, after first reducing the -S-S- bridges with a reducing agent. The number of -S-S- bonds may then be deduced from knowledge of the number of -SH groups in 20 the non-reduced protein relative to the total -SH content after reduction. For example, a method was developed by Cavallini et al (1966), Nature 212,294 where -S-S- groups were reduced with borohydride, and the number of -SH groups formed determined by using the method of Ellman (ibid).

Determination of the position of disulphide bonds  
in proteins

The formation of strongly acidic sulphonic groups by the oxidative cleavage of S-S bonds sharply changes the electrophoretic mobility of peptides containing cysteine. 5 Brown and Hartley (1963,1966) (1963,Biochem.J., 89,59P) (1966,Biochem.J., 101,214) developed a method for locating S-S bridges in proteins using "diagonal electrophoresis" of peptides on paper. An enzymic hydrosylate is subjected to 10 electrophoresis at pH6.5. After drying, a strip is cut out from the electrophore- gram and is sewn onto a new sheet of paper. A second electrophoresis is then carried out at pH6.5 at a right angle to the direction of the first electrophoresis. As a result, the peptides are arranged, 15 according to their mobilities, along a diagonal line. If the strip of paper cut out after the first electrophoresis is exposed to vapours of performic acid, the peptides containing cysteic acid formed by this treatment are located off the diagonal. By this method, one easily identifies the peptides 20 that are linked in a protein by S-S bridges.

Such peptides are eluted from the paper and subjected to amino-acid analysis and/or peptide sequencing and thus the location of peptide bonds in the protein determined.

Example 8Construction of IFNX815, IFNX816, IFNX817, IFNX818, and IFNX457

The DNA nucleotide sequence and amino acid sequence of the modified-interferons IFNX815, 816, 817, 818, and 457 are shown in charts 4-8. These DNA sequences are constructed from the plasmids pJA1, pJA2, and pJA3 utilizing the methods of Examples 3, 4, 5, and 6. The modified interferons are isolated using the methods of Example 9. Alternatively, an entire DNA sequence specified by charts 4-8 is synthesized using the standard genetic code indicating triplet codons and standard phosphotriester chemistry of Examples 3, 4, and 5. Plasmids pMN39-1, pJA1, pJA2, or pJA3 are then utilized to produce the plasmids utilizing the method of Example 6.

Example 915 Interferon Extraction and Purification

The interferons can be isolated from the producing cell by use of the following procedure:

Step 1. Centrifugation of broken cell preparation.

Step 2. Resuspend the pellet in 50mM Tris-Cl pH 8.0 with three-fold w/w/ excess of SDS over protein. Add DTT

20

to 100mM and warm to 95°. Hold at 95° for five minutes.

Step 3. Centrifuge to obtain a clarified extract.

Step 4. Gel filter on an AcA44 column (4.4 X 60 cm) with 50mM  
5 Tris-Cl pH 8.0, 10mM DTT, 0.5% SDS as eluent,  
40ml/hr. This column size will allow the proceeds of  
a nominal 10L fermentation (at the current cell  
density) to be processed in one batch.

#### Parenteral Administration

10 The modified interferons of the present invention can be  
formulated according to methods well known for pharmaceutical  
compositions, wherein the active interferon polypeptide is  
combined in admixture with a pharmaceutically acceptable  
carrier vehicle, such as albumin. Remington's Pharmaceutical  
15 Sciences by E. W. Martin, hereby incorporated by reference,  
describes composition and formulations suitable for delivery of  
the interferons on the present invention.

Pharmaceutical compositions of the present invention will  
contain an effective amount of the interferon protein together  
20 with a suitable carrier allowing therapeutic administration.  
One mode of administration is parenteral, another mode of  
administration is nasal spray.

Administration of the interferon composition is indicated for patients requiring antitumor cell growth or immune modulation, or antiviral treatment. Dosage and dose rate may parallel those now in use in clinical trials of approximately 5  $10^5$  to  $10^8$  units daily. Dosages significantly below or above these levels may be indicated in long term administration or during acute short term treatment. It is anticipated that a preferred dosage rate is  $10^6$ - $10^7$  units daily for parenteral administration.

CLAIMS

1. A modified-interferon molecule comprising the amino acid sequence of a first interferon with one or more cysteine deletions or substitutions at said first interferon sequence positions corresponding to a second interferon's cysteine positions.  
5
2. The modified-interferon of Claim 1 wherein the cysteine deletions or substitutions permit the formation of one or more disulfide linkages at positions corresponding to the disulfide linkages in a second interferon.
- 10 3. The modified-interferon of Claim 2 wherein the first interferon is human beta interferon and the second interferon is a human alpha interferon.
4. The modified-interferon of Claim 2 wherein the first interferon is human alpha interferon and the second  
15 interferon is human beta interferon.
5. The modified-interferon of Claim 2 wherein the first interferon is human beta interferon and the second interferon is human gamma interferon.

6. The modified interferon of Claim 2 wherein the first interferon is human gamma interferon and the second interferon is human beta interferon.
7. The modified-interferon of Claim 2 wherein the first  
5 interferon is a human alpha interferon and the second interferon is a human gamma interferon.
8. The modified-interferon of Claim 2 wherein the first interferon is a human gamma interferon and the second interferon is a human alpha interferon.
- 10 9. The modified-interferon of Claim 3 wherein one of the beta interferon amino acids 1 to 6 are replaced by a cysteine.
10. The modified-interferon of Claim 3 wherein one of the beta interferon amino acids 97 to 105 are replaced by a cysteine.
11. The modified-interferon of Claim 9 wherein one of the beta  
15 interferon amino acids 97 to 105 are replaced by a cysteine.
12. A modified-interferon of Claim 9 wherein the amino acid sequence comprises the sequence of IFNX 802.
13. A modified-interferon of Claim 11 wherein the amino acid sequence comprises the sequence of IFNX 803.

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14. A modified-interferon of Claim 11 wherein the amino acid sequence comprises the sequence of IFNX 804.
15. The modified-interferon of Claim 9 wherein the amino acid sequence comprises the sequence of IFNX 815.
- 5 16. The modified-interferon of Claim 11 wherein the amino acid sequence comprises the sequence of IFNX 816.
17. The modified-interferon of Claim 9 wherein the amino acid sequence comprises the sequence of IFNX 817.
18. The modified-interferon of Claim 11 wherein the amino acid  
10 sequence comprises the sequence of IFNX 818.
19. The modified-interferon of Claim 11 wherein the amino acid sequence comprises the sequence of IFNX 457.
20. The modified-interferon of Claim 11 wherein a disulfide bond is formed between a Cysteine in positions 1 to 6 and a  
15 Cysteine in positions 97 to 105.
21. The modified-interferon of Claim 14 wherein a first disulfide bond is formed between a Cysteine 1 to 6 and a Cysteine 97 to 105 and a second disulfide bound is formed between Cysteine 31 and Cysteine 141.



- 45 -

22. A bacterial extract comprising greater than 90% pure polypeptide consisting essentially of the amino acid sequence of the hybrid or modified interferons according to any one of claims 1-21.
- 5 23. A pharmaceutical composition comprising a therapeutically effective amount of a modified interferon according to any one of claims 1-21, together with a pharmaceutically acceptable diluent.
24. A composition according to claim 23 suitable for parenteral administration.
- 10 25. A hybrid or modified interferon according to any one of claims 1-21 or a composition according to claim 23 or 24 for use in an anti-viral, anti-proliferative, anti-tumour, immunomodulatory or immunogenic method of treatment of the human or animal body.
- 15 26. A process for producing a modified interferon claimed in any one of claims 1-21 which process comprises causing a microorganism, transformed with a replicable microbial expression vehicle capable of expressing said modified interferon and recovering said modified interferon.
- 20 27. A process for producing microorganisms capable of expressing a modified interferon claimed in any one of claims 1-19 comprising: a) transforming a microorganism with a replicable microbial expression vehicle capable of expressing said modified interferon and b) fermenting the
- 25 transformed microorganism.

28. A method of stabilizing a modified interferon comprising the creation of new disulfide bonds.
29. A DNA polymer comprising a nucleotide sequence that encodes for the synthesis of the polypeptide IFNX802, IFNX803, IFNX804, IFNX815, IFNX816, IFNX817, IFNX818 or IFNX457.
30. A plasmid containing the DNA polymer of claim 29.
31. The plasmid of claim 30 comprising the plasmid pJA1 (ATCC 39520).
32. The plasmid of claim 30 comprising the plasmid pJA2 (ATCC 39521).
33. The plasmid of claim 30 comprising the plasmid pJA3 (ATCC 39522).

IFNX 802IFN $\beta$ [Tyr<sup>3</sup> -> Cys<sup>3</sup>]

5 10 15  
 MET-SER-CYS-ASN-LEU-LEU-GLY-PHE-LEU-GLN-ARG-SER-SER-ASN-PHE-  
 ATG AGC TGC AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT  
 20 25 30  
 GLN-CYS-GLN-LYS-LEU-LEU-TRP-GLN-LEU-ASN-GLY-ARG-LEU-GLU-TYR-  
 CAG TGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA TAC  
 35 40 45  
 CYS-LEU-LYS-ASP-ARG-MET-ASN-PHE-ASP-ILE-PRO-GLU-GLU-ILE-LYS-  
 TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG  
 50 55 60  
 GLN-LEU-GLN-GLN-PHE-GLN-LYS-GLU-ASP-ALA-ALA-LEU-THR-ILE-TYR-  
 CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA TTG ACC ATC TAT  
 65 70 75  
 GLU-MET-LEU-GLN-ASN-ILE-PHE-ALA-ILE-PHE-ARG-GLN-ASP-SER-SER-  
 GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT  
 80 85 90  
 SER-THR-GLY-TRP-ASN-GLU-THR-ILE-VAL-GLU-ASN-LEU-LEU-ALA-ASN-  
 AGC ACT GGC TGG AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT  
 95 100 105  
 VAL-TYR-HIS-GLN-ILE-ASN-HIS-LEU-LYS-THR-VAL-LEU-GLU-GLU-LYS-  
 GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA GTC CTG GAA GAA AAA  
 110 120 125  
 LEU-GLU-LYS-GLU-ASP-PHE-THR-ARG-GLY-LYS-LEU-MET-SER-SER-LEU-  
 CTG GAG AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG  
 130 135 140  
 HIS-LEU-LYS-ARG-TYR-TYR-GLY-ARG-ILE-LEU-HIS-TYR-LEU-LYS-ALA-  
 CAC CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG AAG GCC  
 145 150 155  
 LYS-GLU-TYR-SER-HIS-CYS-ALA-TRP-THR-ILE-VAL-ARG-VAL-GLU-ILE-  
 AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC  
 160 165 170  
 LEU-ARG-ASN-PHE-TYR-PHE-ILE-ASN-ARG-LEU-THR-GLY-TYR-LEU-ARG-  
 CTA AGG AAC TTT TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA

ASN-TER-  
 AAC TGA

MSCNLLGFLQRSSNFQCQKLLWQLNGRLEYCLKDRMNFDIPEEIKOLOQFQKEDAALTIY  
 EMLQNI FAIFRQDSSSTGWN ETIVENLLANVYHQINHLKTVLEEKLEKEDFTRGKLMSSL  
 HLKRY YGRILHYLKAKEYSHCAWTIVRVEILRN FYFINRLTG YLRN<

IFNX 803IFN $\beta$ [Tyr<sup>3</sup> -> Cys<sup>3</sup>][Val<sup>101</sup> -> Cys<sup>101</sup>]

5 10 15  
 MET-SER-CYS-ASN-LEU-LEU-GLY-PHE-LEU-GLN-ARG-SER-SER-ASN-PHE-  
 ATG AGC TGC AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT

20 25 30  
 GLN-CYS-GLN-LYS-LEU-LEU-TRP-GLN-LEU-ASN-GLY-ARG-LEU-GLU-TYR-  
 CAG TGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA TAC

35 40 45  
 CYS-LEU-LYS-ASP-ARG-MET-ASN-PHE-ASP-ILE-PRO-GLU-GLU-ILE-LYS-  
 TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG

50 55 60  
 GLN-LEU-GLN-GLN-PHE-GLN-LYS-GLU-ASP-ALA-ALA-LEU-THR-ILE-TYR-  
 CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA TTG ACC ATC TAT

65 70 75  
 GLU-MET-LEU-GLN-ASN-ILE-PHE-ALA-ILE-PHE-ARG-GLN-ASP-SER-SER-  
 GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT

80 85 90  
 SER-THR-GLY-TRP-ASN-GLU-THR-ILE-VAL-GLU-ASN-LEU-LEU-ALA-ASN-  
 AGC ACT GGC TGG AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT

95 100 105  
 VAL-TYR-HIS-GLN-ILE-ASN-HIS-LEU-LYS-THR-CYS-LEU-GLU-GLU-LYS-  
 GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA TGC CTG GAA GAA AAA

110 120 125  
 LEU-GLU-LYS-GLU-ASP-PHE-THR-ARG-GLY-LYS-LEU-MET-SER-SER-LEU-  
 CTG GAG AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG

130 135 140  
 HIS-LEU-LYS-ARG-TYR-TYR-GLY-ARG-ILE-LEU-HIS-TYR-LEU-LYS-ALA-  
 CAC CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG AAG GCC

145 150 155  
 LYS-GLU-TYR-SER-HIS-CYS-ALA-TRP-THR-ILE-VAL-ARG-VAL-GLU-ILE-  
 AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC

160 165 170  
 LEU-ARG-ASN-PHE-TYR-PHE-ILE-ASN-ARG-LEU-THR-GLY-TYR-LEU-ARG-  
 CTA AGG AAC TTT TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA

ASN-TER-  
 AAC TGA

MSCNLLGFLQRSSNFQCQKLLWQLNGRLEYCLKDRMNFDIPEEIKQLQOQFQKEDAALTIY  
 EMLQNIFAIFRQDSSSTGWNETIVENLLANVYHQINHLKTCLEEKLEKEDFTRGKLMSSL  
 HLKRYYGRIHLHYLKAKEYSHCAWTIVRVEILRNFFYFINRLTGYLNR<

IFNX 804

IFN $\beta$ [Tyr<sup>3</sup> -> Cys<sup>3</sup>][Cys<sup>17</sup> -> Ser<sup>17</sup>][Val<sup>101</sup> -> Cys<sup>101</sup>]

MET-SER-CYS-ASN-LEU-LEU-GLY-PHE-LEU-GLN-ARG-SER-SER-ASN-PHE-  
 ATG AGC TGC AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT  
 GLN-SER-GLN-LYS-LEU-LEU-TRP-GLN-LEU-ASN-GLY-ARG-LEU-GLU-TYR-  
 CAG AGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA TAC  
 CYS-LEU-LYS-ASP-ARG-MET-ASN-PHE-ASP-ILE-PRO-GLU-GLU-ILE-LYS-  
 TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG  
 GLN-LEU-GLN-GLN-PHE-GLN-LYS-GLU-ASP-ALA-ALA-LEU-THR-ILE-TYR-  
 CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA TTG ACC ATC TAT  
 GLU-MET-LEU-GLN-ASN-ILE-PHE-ALA-ILE-PHE-ARG-GLN-ASP-SER-SER-  
 GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT  
 SER-THR-GLY-TRP-ASN-GLU-THR-ILE-VAL-GLU-ASN-LEU-LEU-ALA-ASN-  
 AGC ACT GGC TGG AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT  
 VAL-TYR-HIS-GLN-ILE-ASN-HIS-LEU-LYS-THR-CYS-LEU-GLU-GLU-LYS-  
 GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA TGC CTG GAA GAA AAA  
 LEU-GLU-LYS-GLU-ASP-PHE-THR-ARG-GLY-LYS-LEU-MET-SER-SER-LEU-  
 CTG GAG AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG  
 HIS-LEU-LYS-ARG-TYR-TYR-GLY-ARG-ILE-LEU-HIS-TYR-LEU-LYS-ALA-  
 CAC CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG AAG GCC  
 LYS-GLU-TYR-SER-HIS-CYS-ALA-TRP-THR-ILE-VAL-ARG-VAL-GLU-ILE-  
 AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC  
 LEU-ARG-ASN-PHE-TYR-PHE-ILE-ASN-ARG-LEU-THR-GLY-TYR-LEU-ARG-  
 CTA AGG AAC TTT TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA  
 ASN-TER-  
 AAC TGA

MSCNLLGFLQRSSNFQSQKLLWQLNGRLEYCLKDRMNFDIPEEIKQLQQFQKEDAALTIY  
 EMLQNIFAIFRODSSSTGWNETIVENLLANVYHQINHLKTCLEEKLEKEDFTRGKLMSL  
 HLKRYYGRILHYLKAKEYSHCAWTIVRVEILRNIFYFINRLTGYLNR<

IPNX815

IPN- $\beta$  [ser<sup>2</sup>→cys<sup>2</sup>] [cys<sup>17</sup>→ser<sup>17</sup>]

5 10 15  
 MET-CYS-TYR-ASN-LEU-LEU-GLY-PHE-LEU-GLN-ARG-SER-SER-ASN-PHE-  
 ATG TGT TAC AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT

20 25 30  
 GLN-SER-GLN-LYS-LEU-LEU-TRP-GLN-LEU-ASN-GLY-ARG-LEU-GLU-TYR-  
 CAG AGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA TAC

35 40 45  
 CYS-LEU-LYS-ASP-ARG-MET-ASN-PHE-ASP-ILE-PRO-GLU-GLU-ILE-LYS-  
 TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG

50 55 60  
 GLN-LEU-GLN-GLN-PHE-GLN-LYS-GLU-ASP-ALA-ALA-LEU-THR-ILE-TYR-  
 CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA TTG ACC ATC TAT

65 70 75  
 GLU-MET-LEU-GLN-ASN-ILE-PHE-ALA-ILE-PHE-ARG-GLN-ASP-SER-SER-  
 GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT

80 85 90  
 SER-THR-GLY-TRP-ASN-GLU-THR-ILE-VAL-GLU-ASN-LEU-LEU-ALA-ASN-  
 AGC ACT GGC TGG AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT

95 100 105  
 VAL-TYR-HIS-GLN-ILE-ASN-HIS-LEU-LYS-THR-VAL-LEU-GLU-GLU-LYS-  
 GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA GTC CTG GAA GAA AAA

110 115 120  
 LEU-GLU-LYS-GLU-ASP-PHE-THR-ARG-GLY-LYS-LEU-MET-SER-SER-LEU-  
 CTG GAG AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG

125 130 135  
 HIS-LEU-LYS-ARG-TYR-TYR-GLY-ARG-ILE-LEU-HIS-TYR-LEU-LYS-ALA-  
 CAC CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG AAG GCC

140 145 150  
 LYS-GLU-TYR-SER-HIS-CYS-ALA-TRP-THR-ILE-VAL-ARG-VAL-GLU-ILE-  
 AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC

155 160 165  
 LEU-ARG-ASN-PHE-TYR-PHE-ILE-ASN-ARG-LEU-THR-GLY-TYR-LEU-ARG-  
 CTA AGG AAC TTT TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA

ASN-\*\*\*-  
 AAC TGA

10 20 30 40 50  
 MCYNLLGFLQ-RSSNFQSQKL-LWQLNGRLEY-CLKDRMNFDI-PEEIKQLQKF-

60 70 80 90 100  
 QKEDAALTIY-EMLQNI FAIF-RQDSSSTGLN-ETIVENLLAN-VYHQINHLKT-

110 120 130 140 150  
 VLEEKLEKED-FTRGKLMSSL-HLKRY YGRIL-HYLKAKEYSH-CAWTIURVEI-

## IFNX816

IFN- $\beta$ [ser<sup>2</sup>→cys<sup>2</sup>][cys<sup>17</sup>→ser<sup>17</sup>][val<sup>101</sup>→cys<sup>101</sup>]

5 10 15  
 MET-CYS-TYR-ASN-LEU-LEU-GLY-PHE-LEU-GLN-ARG-SER-SER-ASN-PHE-  
 ATG TGT TAC AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT  
 20 25 30  
 GLN-SER-GLN-LYS-LEU-LEU-TRP-GLN-LEU-ASN-GLY-ARG-LEU-GLU-TYR-  
 CAG AGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA TAC  
 35 40 45  
 CYS-LEU-LYS-ASP-ARG-MET-ASN-PHE-ASP-ILE-PRO-GLU-GLU-ILE-LYS-  
 TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG  
 50 55 60  
 GLN-LEU-GLN-GLN-PHE-GLN-LYS-GLU-ASP-ALA-ALA-LEU-THR-ILE-TYR-  
 CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA TTG ACC ATC TAT  
 65 70 75  
 GLU-MET-LEU-GLN-ASN-ILE-PHE-ALA-ILE-PHE-ARG-GLN-ASP-SER-SER-  
 GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT  
 80 85 90  
 SER-THR-GLY-TRP-ASN-GLU-THR-ILE-VAL-GLU-ASN-LEU-LEU-ALA-ASN-  
 AGC ACT GGC TGG AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT  
 95 100 105  
 VAL-TYR-HIS-GLN-ILE-ASN-HIS-LEU-LYS-THR-CYS-LEU-GLU-GLU-LYS-  
 GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA TGC CTG GAA GAA AAA  
 110 115 120  
 LEU-GLU-LYS-GLU-ASP-PHE-THR-ARG-GLY-LYS-LEU-MET-SER-SER-LEU-  
 CTG GAG AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG  
 125 130 135  
 HIS-LEU-LYS-ARG-TYR-TYR-GLY-ARG-ILE-LEU-HIS-TYR-LEU-LYS-ALA-  
 CAC CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG AAG GCC  
 140 145 150  
 LYS-GLU-TYR-SER-HIS-CYS-ALA-TRP-THR-ILE-VAL-ARG-VAL-GLU-ILE-  
 AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC  
 155 160 165  
 LEU-ARG-ASN-PHE-TYR-PHE-ILE-ASN-ARG-LEU-THR-GLY-TYR-LEU-ARG-  
 CTA AGG AAC TTT TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA

ASN-\*\*\*-  
 AAC TGA

10 20 30 40 50  
 MCYNLLGFLQ-RSSNFQSQKL-LWQLNGRLEY-CLKDRMNFDI-PEEI KQLQGF-  
 60 70 80 90 100  
 QKEDAALTIY-EMLQNI FAIF-RQDSSSTGLN-ETIVENLLAN-VYHQINHLKT-  
 110 120 130 140 150  
 CLEEKLEKED-FTRGKLMSSL-HLKRY YGRIL-HYLKAK EYSH-CAWTIURVEI-  
 160  
 LRNFYFINRL-TGYLRN<

IPNX817

IFN- $\beta$ [ser<sup>2</sup>->cys<sup>2</sup>]

5 10 15  
 MET-CYS-TYR-ASN-LEU-LEU-GLY-PHE-LEU-GLN-ARG-SER-SER-ASN-PHE-  
 ATG TGT TAC AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT

20 25 30  
 GLN-CYS-GLN-LYS-LEU-LEU-TRP-GLN-LEU-ASN-GLY-ARG-LEU-GLU-TYR-  
 CAG TGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA TAC

35 40 45  
 CYS-LEU-LYS-ASP-ARG-MET-ASN-PHE-ASP-ILE-PRO-GLU-GLU-ILE-LYS-  
 TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG

50 55 60  
 GLN-LEU-GLN-GLN-PHE-GLN-LYS-GLU-ASP-ALA-ALA-LEU-THR-ILE-TYR-  
 CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA TTG ACC ATC TAT

65 70 75  
 GLU-MET-LEU-GLN-ASN-ILE-PHE-ALA-ILE-PHE-ARG-GLN-ASP-SER-SER-  
 GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT

80 85 90  
 SER-THR-GLY-TRP-ASN-GLU-THR-ILE-VAL-GLU-ASN-LEU-LEU-ALA-ASN-  
 AGC ACT GGC TGG AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT

95 100 105  
 VAL-TYR-HIS-GLN-ILE-ASN-HIS-LEU-LYS-THR-VAL-LEU-GLU-GLU-LYS-  
 GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA GTC CTG GAA GAA AAA

110 115 120  
 LEU-GLU-LYS-GLU-ASP-PHE-THR-ARG-GLY-LYS-LEU-MET-SER-SER-LEU-  
 CTG GAG AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG

125 130 135  
 HIS-LEU-LYS-ARG-TYR-TYR-GLY-ARG-ILE-LEU-HIS-TYR-LEU-LYS-ALA-  
 CAC CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG AAG GCC

140 145 150  
 LYS-GLU-TYR-SER-HIS-CYS-ALA-TRP-THR-ILE-VAL-ARG-VAL-GLU-ILE-  
 AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC

155 160 165  
 LEU-ARG-ASN-PHE-TYR-PHE-ILE-ASN-ARG-LEU-THR-GLY-TYR-LEU-ARG-  
 CTA AGG AAC TTT TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA

ASN-\*\*\*-  
AAC TGA

10 20 30 40 50  
 MCYNLLGFLQ-RSSNFQCQKL-LWQLNGRLEY-CLKDRMNFDI-PEEIKQLQQF-

60 70 80 90 100  
 QKEDAALTIY-EMLNIFAIF-RQDSSSTGLN-ETIVENLLAN-VYHQINHLKT-

110 120 130 140 150  
 VLEEKLEKED-FTRGKLMSSL-HLKRYYGRIL-HYLKKEYSH-CAWTJURVEI-

160  
 LRNFYFINRL-TGYLRN<



## IPNX818

IPN- $\beta$ [ser<sup>2</sup>→cys<sup>2</sup>][val<sup>181</sup>→cys<sup>181</sup>]

5 10 15  
 MET-CYS-TYR-ASN-LEU-LEU-GLY-PHE-LEU-GLN-ARG-SER-SER-ASN-PHE-  
 ATG TGT TAC AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT  
 20 25 30  
 GLN-CYS-GLN-LYS-LEU-LEU-TRP-GLN-LEU-ASN-GLY-ARG-LEU-GLU-TYR-  
 CAG TGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA TAC  
 35 40 45  
 CYS-LEU-LYS-ASP-ARG-MET-ASN-PHE-ASP-ILE-PRO-GLU-GLU-ILE-LYS-  
 TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG  
 50 55 60  
 GLN-LEU-GLN-GLN-PHE-GLN-LYS-GLU-ASP-ALA-ALA-LEU-THR-ILE-TYR-  
 CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA TTG ACC ATC TAT  
 65 70 75  
 GLU-MET-LEU-GLN-ASN-ILE-PHE-ALA-ILE-PHE-ARG-GLN-ASP-SER-SER-  
 GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT  
 80 85 90  
 SER-THR-GLY-TRP-ASN-GLU-THR-ILE-VAL-GLU-ASN-LEU-LEU-ALA-ASN-  
 AGC ACT GGC TGG AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT  
 95 100 105  
 VAL-TYR-HIS-GLN-ILE-ASN-HIS-LEU-LYS-THR-CYS-LEU-GLU-GLU-LYS-  
 GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA TGC CTG GAA GAA AAA  
 110 115 120  
 LEU-GLU-LYS-GLU-ASP-PHE-THR-ARG-GLY-LYS-LEU-MET-SER-SER-LEU-  
 CTG GAG AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG  
 125 130 135  
 HIS-LEU-LYS-ARG-TYR-TYR-GLY-ARG-ILE-LEU-HIS-TYR-LEU-LYS-ALA-  
 CAC CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG AAG GCC  
 140 145 150  
 LYS-GLU-TYR-SER-HIS-CYS-ALA-TRP-THR-ILE-VAL-ARG-VAL-GLU-ILE-  
 AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC  
 155 160 165  
 LEU-ARG-ASN-PHE-TYR-PHE-ILE-ASN-ARG-LEU-THR-GLY-TYR-LEU-ARG-  
 CTA AGG AAC TTT TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA

ASN-\*\*\*-  
AAC TGA

10 20 30 40 50  
 MCYNLLGFLQ-RSSNFQCQKL-LWQLNGRLEY-CLKDRMNFDI-PEEIQLQKQF-  
 60 70 80 90 100  
 QKEDAALTIY-EMLQNI FAIF-RQDSSSTGWN-ETIVENLLAN-VYHQINHLKT-  
 110 120 130 140 150  
 CLEEKLEKED-FTRGKLMSSL-HLKRYYGRIL-HYLKAKEYSH-CAWTIVRVEI-  
 160  
 LRNFYF INRL-TGYLRN<

IFN- $\beta$ [IFN- $\beta$ 2-7 $\rightarrow$ IFN- $\alpha$ 21-5][cys<sup>16</sup> $\rightarrow$ ser<sup>16</sup>][val<sup>100</sup> $\rightarrow$ cys<sup>100</sup>]

5 10 15  
 MET-CYS-ASP-LEU-PRO-GLN-PHE-LEU-GLN-ARG-SER-SER-ASN-PHE-GLN-  
 ATG TGC GAC TTA CCA CAA TTC CTA CAA AGA AGC AGC AAT TTT CAG  
 20 25 30  
 SER-GLN-LYS-LEU-LEU-TRP-GLN-LEU-ASN-GLY-ARG-LEU-GLU-TYR-CYS-  
 TCT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA TAT TGC  
 35 40 45  
 LEU-LYS-ASP-ARG-MET-ASN-PHE-ASP-ILE-PRO-GLU-GLU-ILE-LYS-GLN-  
 CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG CAG  
 50 55 60  
 LEU-GLN-GLN-PHE-GLN-LYS-GLU-ASP-ALA-ALA-LEU-THR-ILE-TYR-GLU-  
 CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA TTG ACC ATC TAT GAG  
 65 70 75  
 MET-LEU-GLN-ASN-ILE-PHE-ALA-ILE-PHE-ARG-GLN-ASP-SER-SER-SER-  
 ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT AGC  
 80 85 90  
 THR-GLY-TRP-ASN-GLU-THR-ILE-VAL-GLU-ASN-LEU-LEU-ALA-ASN-VAL-  
 ACT GGC TGG AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT GTC  
 95 100 105  
 TYR-HIS-GLN-ILE-ASN-HIS-LEU-LYS-THR-CYS-LEU-GLU-GLU-LYS-LEU-  
 TAT CAT CAG ATA AAC CAT CTG AAG ACA TGC CTG GAA GAA AAA CTG  
 110 115 120  
 GLU-LYS-GLU-ASP-PHE-THR-ARG-GLY-LYS-LEU-MET-SER-SER-LEU-HIS-  
 GAG AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG CAC  
 125 130 135  
 LEU-LYS-ARG-TYR-TYR-GLY-ARG-ILE-LEU-HIS-TYR-LEU-LYS-ALA-LYS-  
 CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG AAG GCC AAG  
 140 145 150  
 GLU-TYR-SER-HIS-CYS-ALA-TRP-THR-ILE-VAL-ARG-VAL-GLU-ILE-LEU-  
 GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC CTA  
 155 160 165  
 ARG-ASN-PHE-TYR-PHE-ILE-ASN-ARG-LEU-THR-GLY-TYR-LEU-ARG-ASN-  
 AGG AAC TTT TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA AAC

\*\*\*-  
TGA

10 20 30 40 50  
 MCDLPQFLQR-SSNFQSQKLL-WQLNGRLEYC-LKDRMNF DIP-EEIKQLQQFQ-  
 60 70 80 90 100  
 KEDAALTIYE-MLQNI FAIFR-QDSSSTG WNE-TIVENLLANV-YHQINHLKTC-  
 110 120 130 140 150  
 LEEKLEKEDF-TRGKLMSSLH-LKRY YGRILH-YLKAKEYSHC-AWTIVRVEIL-  
 160  
 RNFYFINRLT-GYLRN<

## SUMMARY FLOWCHART - FORMATION OF IFNX801, X803, X804

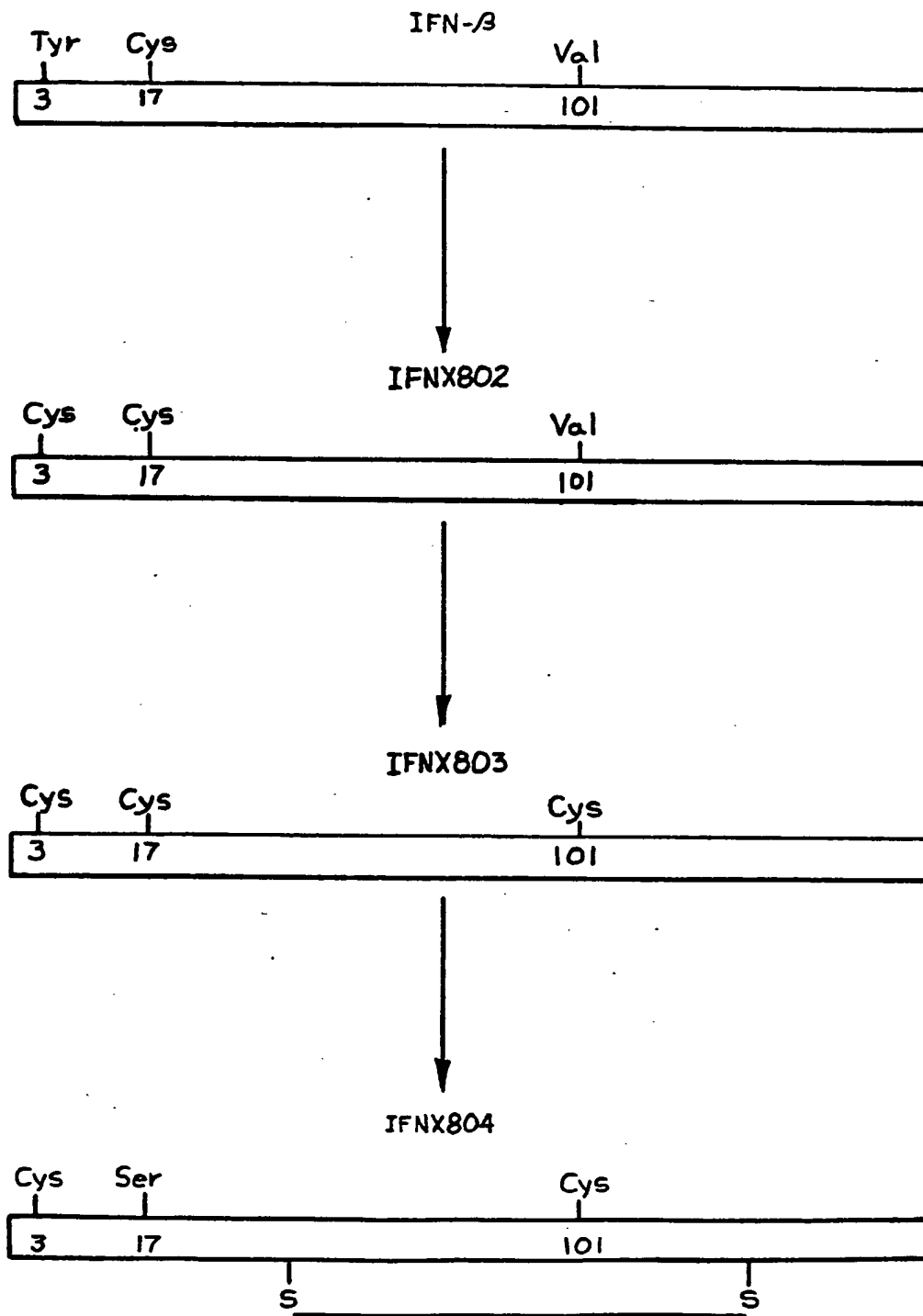


FIG. 1

RESTRICTION MAP, CONSTRUCTION I  
REPLICATIVE FORM SIZE 8.4kbp

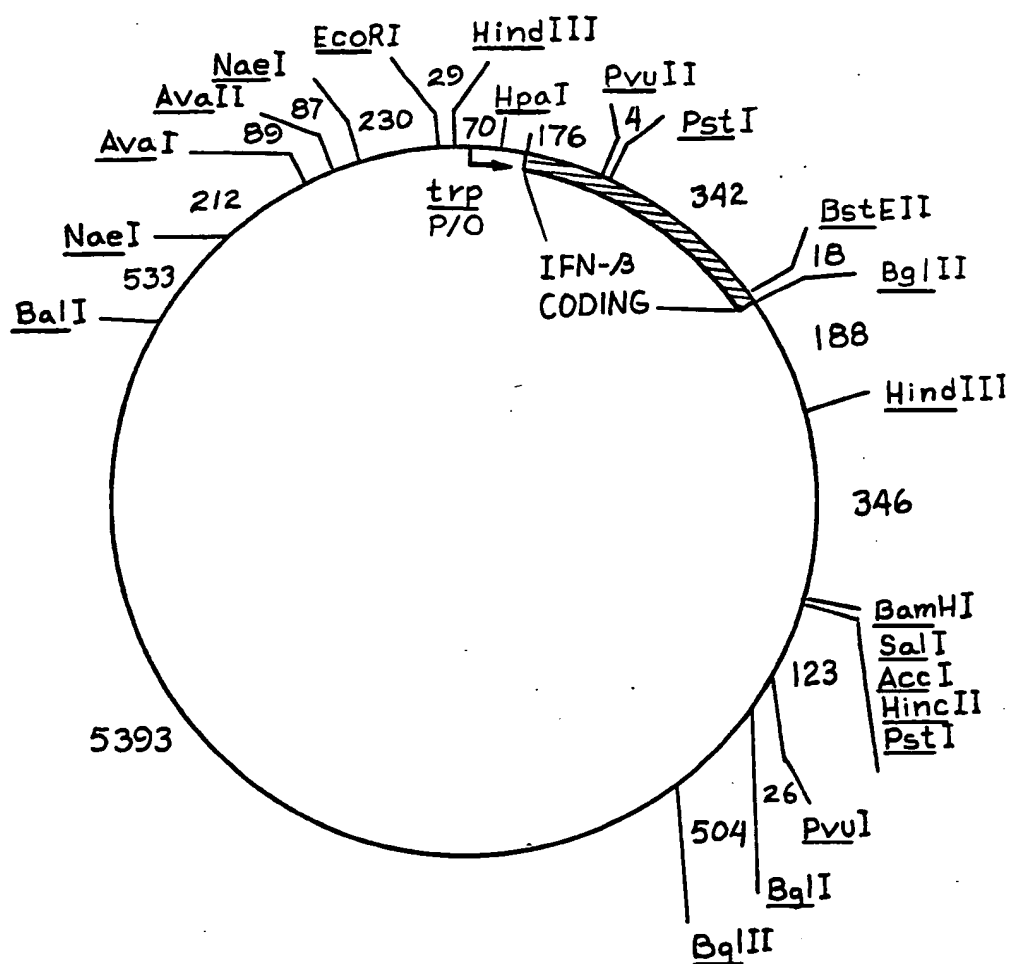
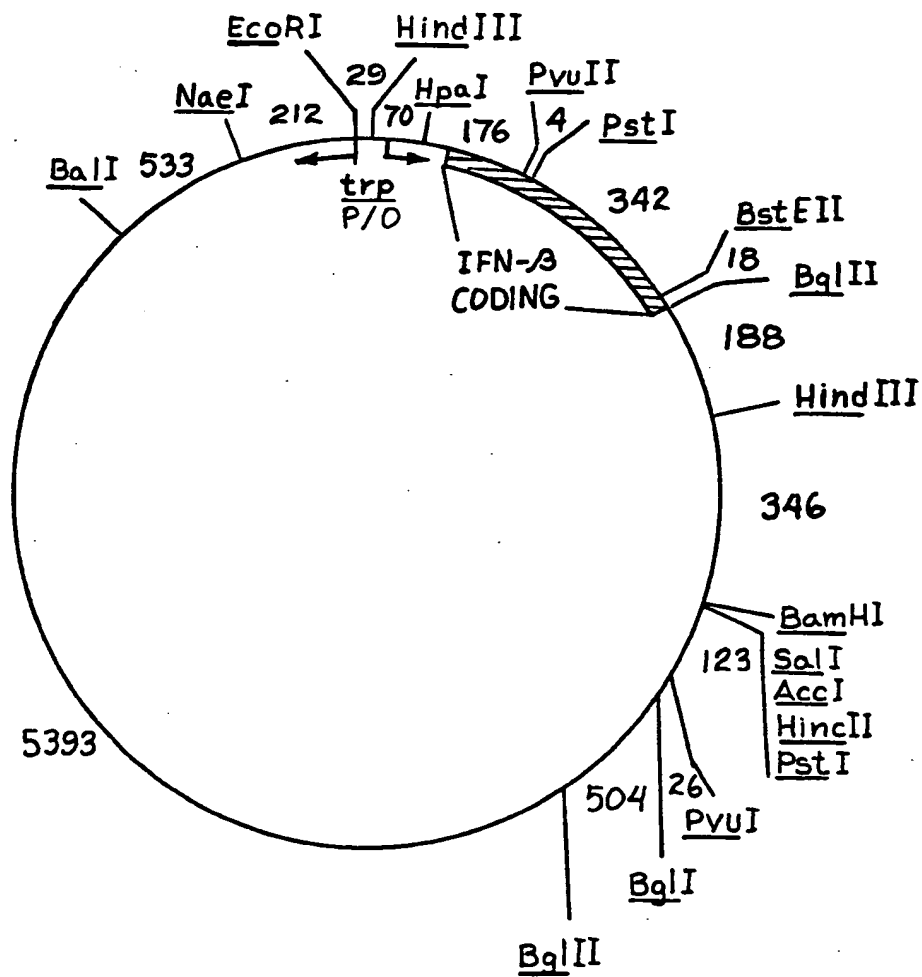


FIG. 2

RESTRICTION MAP, CONSTRUCTIONS II-V  
 REPLICATIVE FORM SIZE 8 kbp.



APPLICABLE TO M13-4AB.00  
 MJA1  
 MJA2  
 MJA3

FIG. 3

SITE DIRECTED CHANGES

1. Amino Acid 3. Human IFN- $\beta$ , Tyr  $\rightarrow$  Cys  
 Product IFNX802 (HuIFN- $\beta$ (Tyr<sup>3</sup>  $\rightarrow$  Cys<sup>3</sup>))

		1 Met	Ser	3 Tyr	Asn	5 Leu	
Original Sequence 5'	TCC	ATG 1	AGC	TAC —	AAC 10	TTG	3'
Primer	3'G	TAC	TCG	ACG —	TTG	A	5'
New Sequence	TCC	ATG	AGC	TGC — Cys	AAC	TTG	

FIG. 4



SITE DIRECTED CHANGES

2. Amino Acid 101. Human IFNX802, Val → Cys

Product IFNX803

(HuIFN- $\beta$ (Tyr<sup>3</sup> → Cys<sup>3</sup>) (Val<sup>101</sup> → Cys<sup>101</sup>))

	98 Leu	Lys	100 Thr	Val	102 Leu	Glu	104 Glu	
Original Sequence 5'	CTG	AAG	ACA	<u>GTC</u>	CTG	GAA	GAA	3'
Primer	AC	TTC	TGT	<u>ACG</u>	GAC	CTT	C	5'
New Sequence	CTG	AAG	ACA	<u>TGC</u> Cys	CTG	GAA	GAA	

FIG. 5



SITE DIRECTED CHANGES

3. Amino Acid 17. Human IFNX803 Cys → Ser

Product IFNX804

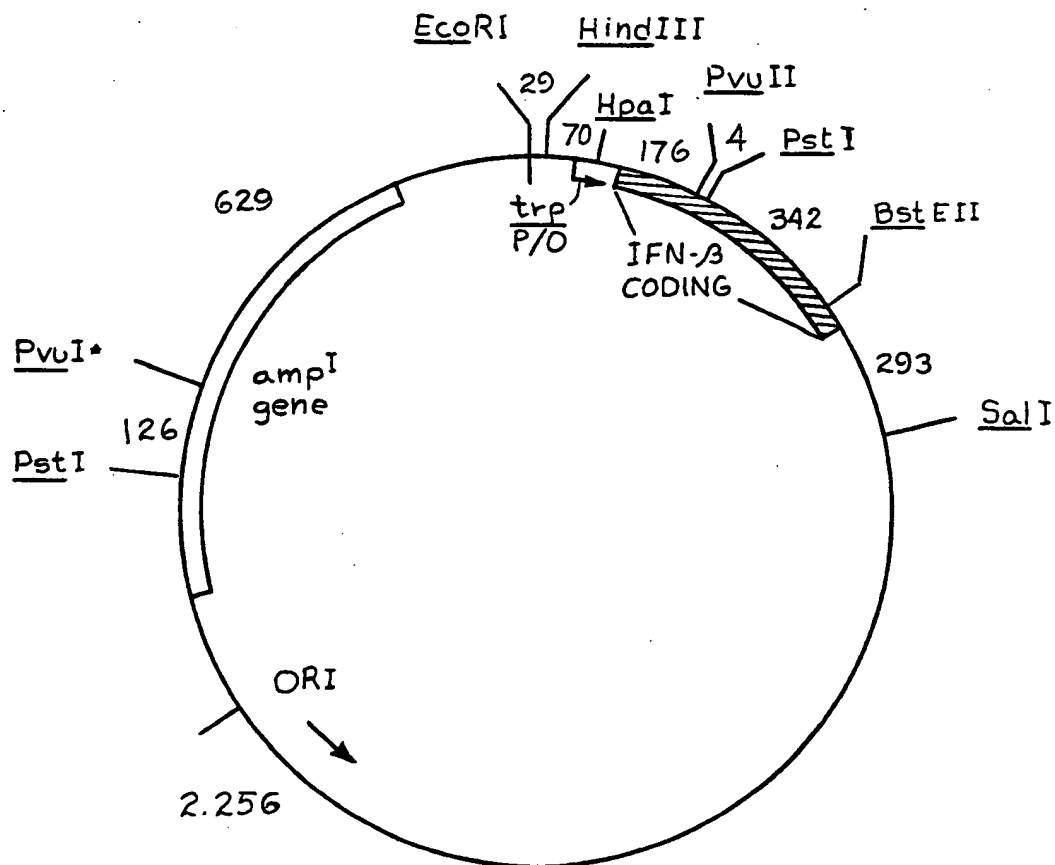
(HuIFN- $\beta$ (Tyr<sup>3</sup> → Cys<sup>3</sup>)(Val<sup>101</sup> → Cys<sup>101</sup>)(Cys<sup>17</sup> → Ser<sup>17</sup>))

	13 Ser	Asn	15 Phe	Gln	17 Cys	Gln	
Original Sequence 5'	AGC	AAT	TTT	CAG	TGT	CAG	3'
Primer	G	TTA	AAA	GTC	TCA	GTC	5'
New Sequence	AGC	AAT	TTT	CAG	AGT	CAG	
					Ser		

FIG. 6





RESTRICTION MAP, CONSTRUCTIONS VI, VII, VIIIPLASMID 3.92 kbp.

APPLICABLE TO PMN39-1  
PJA1  
PJA2  
PJA3

**FIG. 7**

(19)



Europäisches Patentamt  
European Patent Office  
Office européen des brevets

(11) Publication number:

**0 237 019**  
**A2**

(12)

# EUROPEAN PATENT APPLICATION

(21) Application number: 87103406.2

(51) Int. Cl.<sup>3</sup>: **C 12 P 21/02**  
**C 12 N 15/00, C 07 K 15/26**  
**C 12 N 5/00, C 12 N 1/20**

(22) Date of filing: 10.03.87

(30) Priority: 14.03.86 JP 54650/86

(43) Date of publication of application:  
16.09.87 Bulletin 87/38

(84) Designated Contracting States:  
CH DE FR GB IT LI

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(64) Interferon conjugate and production thereof using recombinant gene.

(57) An interferon conjugates comprising in a single molecule a region exhibiting biological activities of interferon- $\beta$  and a region exhibiting biological activities of interferon- $\gamma$ ; deoxyribonucleotide sequences coding for the interferon conjugate; recombinant DNAs comprising the deoxyribonucleotide sequence and a deoxyribonucleotide sequence coding for a control region for expression of the interferon conjugate wherein the latter sequence is upstream from the former sequence; transformant organisms transformed with the recombinant DNA; and a process for production of the above-mentioned interferon conjugate comprising the steps of culturing the transformant organism to produce the interferon conjugate, and recovering the interferon conjugate.

EP U 237 019 A2

INTERFERON CONJUGATE AND PRODUCTION THEREOF  
USING RECOMBINANT GENE

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to interferon conjugates comprising interferon- $\beta$  and interferon- $\gamma$ , a process for production of these interferon conjugates, and a gene system including a nucleotide sequence coding for these interferon conjugate, a recombinant DNA comprising the nucleotide sequence, and microorganisms transformed with the recombinant DNA.

2. Description of the Related Art

Interferons are proteins exhibiting various biological activities, such as antiviral activity, and therefore, are promising for clinical application.

Interferons are classified into three types, i.e., interferon- $\alpha$ , - $\beta$ , and - $\gamma$ , depending on their inducers, producer cells or antigenecities thereof, and are different in structure gene coding therefor, properties as protein, and in biological activities (Interferon no Kagaku (Science of Interferon), edited by Shigeyasu Kobayashi, Kodan Sha).

Interferon- $\beta$  (IFN- $\beta$ ) is a glycoprotein usually produced by fibroblast induced with virus or double-stranded RNA, is stable to treatment at pH 2, and unstable to treatment at 56°C. Gene coding for interferon- $\beta$  has been already isolated (Taniguchi et al., Proc. Jpn. Acad., 55, Ser. B, 464-468, 1979). The nucleotide sequence of the gene and a corresponding amino acid sequence have been determined, and moreover, a process for the production of interferon- $\beta$  using cDNA thus obtained and E. coli as a host has been developed (Taniguchi et al., Proc. Natl. Acad. Sci. USA, 77, 5230-5233, 1980; Goeddel et al., Nucleic Acids Res., 8, 4057-4074, 1980; and Derynck et al., Nature, 287,

193-197, 1980).

Interferon- $\gamma$  (IFN- $\gamma$ ) is a glycoprotein usually produced by T lymphocyte induced with a mitogen, and is unstable to treatment at pH 2. A gene coding for  
5 interferon- $\gamma$  has been also isolated, the nucleotide sequence thereof has been determined, and a process for the production of interferon- $\gamma$  using E. coli as a host has been established (Devos et al., Nucleic Acids Res. 10, 2487-2501, 1982; and Gray et al., Nature, 295,  
10 503-508, 1982). Moreover, an amino acid sequence of native interferon- $\gamma$  has been reported (Rinderknecht et al., J. Biol. Chem., 259, 6790-6797, 1984).

Among the interferons- $\alpha$ , - $\beta$ , and - $\gamma$ , interferons- $\alpha$  and - $\beta$  have been known as interferon I,  
15 and it has been suggested that these interferons have a high structural analogy showing a 29% amino acid sequence homology (Taniguchi et al., Gene, 10, 11-15, 1980), and it is believed that they recognize same receptor. Therefore, the coexistence of interferons- $\alpha$   
20 and - $\beta$  provides their activities only in an additive manner. On the contrary, interferon- $\gamma$ , known as interferon II, has a low amino acid homology with interferon I. It is believed that interferons I and II recognize different receptors (Branca et al., Nature,  
25 294, 768-770, 1981). Therefore, interferons I and II exhibit different spectra of the anti-cell proliferation effect (Interferon no Kagaku (Science of Interferon), edited by Shigeyasu Kobayashi, Kodan Sha, Japan, 22-68, 1985), and exhibit their activities in a synergistic  
30 manner (Czarniecki et al., J. Virol., 49, 490-496, 1984; Fleishmann Jr. et al., J. IFN. Res., 4, 265-274, 1984; and Japanese Unexamined Patent Publication 59-98019).

Although a mixture of interferons- $\beta$  and - $\gamma$  will show the above-mentioned synergism in vitro, it is  
35 doubtful whether such synergism is established in vivo because there is some possibility that both interferon- $\beta$  and - $\gamma$  would not be simultaneously present at a target

site due to their different in vivo pharmacodynamics.

To resolve the above-mentioned disadvantage provided by the problems of in vivo pharmacodynamics, it would be effective to link interferons- $\beta$  and - $\gamma$  to make  
5 a single polypeptide which could exhibit in vivo the same synergism as shown in vitro by a mixture of interferons- $\beta$  and - $\gamma$ . This means that, since a linked polypeptide molecule exhibits the same synergistically enhanced actions as shown by the parent two interferon  
10 molecules, i.e., - $\beta$  and - $\gamma$ , the activity per a molecule is enhanced in comparison to the activity per molecule of the parent interferons, making it possible to obtain a new type of interferon with a higher activity.

Moreover, by causing a single polypeptide to  
15 exhibit the different kinds of actions originally exhibited separately by interferons- $\beta$  and - $\gamma$ , it would be possible to obtain a new type of interferon having a broader spectrum of actions.

Some examples are already known wherein two  
20 polypeptides having different actions are linked to make a single polypeptide having two actions (Yournio et al., Nature, 228, 820-824, 1970; Neuberger et al., Nature, 312, 604-608, 1984; and Bulow et al., Biotechnology, 3, 821-823, 1985). Moreover, insulin molecules were linked  
25 to make a stable polypeptide (Shen and Shi-Hsiang, Proc. Natl. Acad. Sci. USA, 81, 4627-4631, 1984). As another example, there was disclosed a linkage of interferon- $\gamma$  and interleukin-2 to make a single polypeptide which exhibits both actions of interferon- $\gamma$  and interleukin-2  
30 (Japanese Unexamined Patent Publication No. 60-241890).

However, so far there has been no attempt to link interferon- $\beta$  with interferon- $\gamma$  to prepare a single polypeptide having at the same time both a broader spectrum of actions and higher activities.

#### 35 SUMMARY OF THE INVENTION

Therefore, the present invention relates to interferon conjugates comprising interferon- $\beta$  and

interferon- $\gamma$ , which have a broader spectrum of biological actions, such as antiviral action, anti-cell proliferation action, and the like, that were originally carried separately by parent polypeptides, i.e.,

5 interferon- $\beta$  and interferon- $\gamma$ , and which exhibits in vivo synergistically enhanced activities as shown in vivo by a mixture of interferons- $\beta$  and - $\gamma$ .

More specifically, the present invention provides an interferon conjugate comprising in a single molecule  
10 a region exhibiting biological activities of interferon- $\beta$  and a region exhibiting biological activities of interferon- $\gamma$ .

The present invention also provides a deoxyribonucleotide sequence coding for this interferon conjugate.

15 Moreover, the present invention provides a recombinant DNA comprising the above deoxyribonucleotide sequence and a deoxyribonucleotide sequence coding for a control region for expression of the interferon conjugate, wherein the latter sequence is present  
20 upstream from the former sequence.

Moreover, the present invention provides a transformant organism transformed with the recombinant DNA.

Moreover, the present invention provides a process for production of the above-mentioned interferon  
25 conjugate comprising the steps of culturing the transformant organism to produce the interferon conjugate, and recovering the interferon conjugate.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 represents an embodiment of an amino acid  
30 sequence of human mature interferon- $\beta$ ;

Fig. 2 represents an embodiment of an amino acid sequence of human mature interferon- $\gamma$ ;

Fig. 3 represents a structure of plasmid pKM6 for expression of human interferon- $\beta$ ;

35 Fig. 4 represents a structure of plasmid p6hu $\gamma$ -N1 for expression of human interferon- $\gamma$ ;

Fig. 5 represents a structure of plasmid pKM6-Cxho

into which an XhoI site has been introduced at the 3'-terminus of the human interferon - $\beta$  structural gene;

Fig. 6 represents a structure of plasmid p6hu $\gamma$ N1-CKpn into which a KpnI site has been introduced at the 3'-terminus of the human interferon- $\gamma$  structural gene;

Fig. 7 represents a structure of plasmid p6hu $\gamma$ N1ABS-NHin into which a HinPI site has been introduced to take out a human interferon- $\gamma$  structural gene;

Fig. 8 schematically represents a process for construction of a plasmid for expression of an interferon- $\gamma$ - $\beta$  conjugate;

Fig. 9 schematically represents a process for construction of a plasmid for expression of an interferon- $\beta$ - $\gamma$  conjugate;

Fig. 10 schematically represents a process for construction of a plasmid for expression of an interferon- $\gamma$ - $\beta$  conjugate;

Fig. 11 represents a nucleotide sequence coding for an amino acid sequence of a spacer peptide;

Fig. 12 schematically represents a process for construction of a plasmid for expression of an interferon- $\beta$ - $\gamma$  conjugate;

Fig. 13 schematically represents a process for construction of a plasmid for expression of an interferon- $\gamma$ - $\beta$  conjugate in animal cells; and

Fig. 14 schematically represents a process for construction of a plasmid for expression of an interferon- $\gamma$ - $\beta$  conjugate in animal cells.

In Figures 3 to 10 and 12, the numbers 1, 2, and 3 represent a human interferon- $\beta$  structural gene, a human interferon- $\gamma$  structural gene, and a non-coding region of human interferon- $\gamma$  cDNA, respectively.

#### DESCRIPTION OF THE PREFERRED EMBODIMENT

The interferon conjugate of the present invention comprises a region exhibiting biological activities of interferon- $\beta$  and a region exhibiting biological

activities of interferon- $\gamma$  linked directly or via a spacer. According to the present invention, the terms "interferon- $\beta$ " and "interferon- $\gamma$ " include any proteins having activities specific to interferon- $\beta$  and interferon- $\gamma$ , respectively. For example, for interferon- $\gamma$ , a modified interferon- $\gamma$  wherein its polypeptide has been extended by three amino acid residues at N-terminal (Gray et al., Nature, 295, 503-508, 1982), and a modified interferon- $\gamma$  wherein a part of the C-terminal of the polypeptide thereof has been deleted (Rose et al., Biochem. J., 215, 273, 1983), are known. These modified interferon- $\gamma$  fall under the scope of the present invention provided that they maintain interferon activities. Moreover, modified interferon- $\gamma$  wherein some amino acid residues have been replaced by other amino acid residues are disclosed (Japanese Unexamined Patent Publications No. 59-93093 and No. 59-167596). These modified interferon- $\gamma$  also fall under the scope of the present interferon- $\gamma$  provided that they maintain interferon activities. Interferon- $\beta$  also can be modified by the addition, deletion or replacement of one or more amino acid residues as described for interferon- $\gamma$ . Preferably, interferon- $\beta$  comprises a polypeptide having the amino acid sequence shown in Fig. 1, and interferon- $\gamma$  comprises a polypeptide having the amino acid sequence shown in Fig. 2.

Juxtaposition order of interferons- $\beta$  and - $\gamma$  in the present interferon conjugate is not limited. That is, the interferon conjugate can contain as its N-terminal half a polypeptide of interferon- $\beta$  and as its C-terminal half a polypeptide of interferon- $\gamma$ , and vice versa.

In junction region in an interferon conjugate, a polypeptide of interferon- $\beta$  and that of interferon- $\gamma$  can be linked directly, or indirectly via a spacer, such as a peptide. The spacer peptide is preferably a peptide comprising many hydrophilic amino acids, as shown in an example for an enzyme wherein subunits of  $\beta$ -galactosidase



are linked via a spacer peptide (Kushinke et al, EMBO J., 4, 1067-1073, 1985). Moreover, a polypeptide which links domains of a naturally occurring protein can be used as a spacer peptide of the present invention. A  
5 spacer peptide usually consists of up to 50 amino acids. A preferred spacer peptide is, for example, a peptide known as a switch-peptide of immunoglobulin molecule, and also a peptide having the amino acid sequence:  
Thr-Gln-Leu-Gly-Gln-Pro-Lys-Ala-Ala-Lys-Ser-Val-Thr.

10 In the present invention, a structure comprising a polypeptide of interferon- $\beta$  and a polypeptide of interferon- $\gamma$  is designated as "interferon conjugate". More specifically, a structure comprising as its N-terminal half a polypeptide of interferon- $\beta$  and as  
15 its C-terminal half a polypeptide of interferon- $\gamma$  is designated as "interferon- $\beta \cdot \gamma$  conjugate" (IFN- $\beta \cdot \gamma$ ); and a structure comprising as its N-terminal half a polypeptide of interferon- $\gamma$ , and at its C-terminal half, a polypeptide of interferon- $\beta$ , is designated as  
20 "interferon- $\gamma \cdot \beta$  conjugate" (IFN- $\gamma \cdot \beta$ ). On the other hand, a structure comprising as its N-terminal half a polypeptide of interferon- $\beta$  and as its C-terminal half a polypeptide of interferon- $\gamma$  wherein both polypeptides are linked via a spacer is designated as an "interferon- $\beta \gamma$  conjugate" (IFN- $\beta \gamma$ ); and a structure comprising as  
25 its N-terminal half a polypeptide of interferon- $\gamma$  and as its C-terminal half a polypeptide of interferon- $\beta$  wherein both polypeptides are linked via a spacer is designated as an "interferon- $\gamma \beta$  conjugate" (IFN- $\gamma \beta$ ).

30 As processes of the production of an interferon conjugate, there are a chemical synthesis wherein amino acids are coupled in a predetermined order, and genetic engineering process wherein a DNA coding for a target polypeptide is designed and constructed and the target  
35 polypeptide is expressed in appropriate transformed cells. In the present invention, preferably the genetic engineering is used because it enables an easy production

of a target polypeptide, though the means of obtaining a target polypeptide is not limited to genetic engineering.

In the use of genetic engineering for the production of an interferon conjugate, a DNA fragment comprising  
5 a nucleotide sequence coding for a polypeptide of interferon- $\beta$  and a nucleotide sequence coding for a polypeptide of interferon- $\beta$ , wherein these nucleotide sequences are directly linked or indirectly linked via a  
10 nucleotide sequence coding for a spacer peptide, are prepared, and this DNA fragment is linked to appropriate control regions for expression of the target polypeptide, and the coding sequence is expressed in appropriate host cells.

Any nucleotide sequence coding for an interferon  
15 conjugate can be used. That is, if more than one codon is present for an amino acid, any codon which codes for the amino acid can be used. Preferably, the same nucleotide sequences as those of the cDNAs of  
20 interferons- $\beta$  and - $\gamma$  respectively are used (Taniguchi et al., *Gene*, 10, 11-15, 1980; and Devos et al., *Nucleic Acids Res.*, 10, 2487-2501, 1982).

To obtain a DNA fragment having a nucleotide sequence coding for an interferon conjugate, chemical synthesis of the DNA, isolation and linking of genes  
25 coding for interferons- $\beta$  and - $\gamma$ , and a combination of such means can be used. The chemical synthesis of a DNA fragment having a desired nucleotide sequence can be achieved according to a conventional procedure (Edge et al., *Nature*, 292, 756-762, 1981; and Tanaka et al.,  
30 *Nucleic Acids Res.*, 11, 1707-1723, 1983). DNA fragment coding for interferon- $\beta$  or - $\gamma$  can be derived from chromosomal DNA, or preferably, prepared as cDNA. cDNA can be isolated according to a known procedure  
(Taniguchi et al., *Proc. Jpn. Acad.*, 55, Ser. B, 464,  
35 1979; Goeddel et al., *Nucleic Acids Res.*, 8, 4057-4074, 1980; Derynck et al., *Nature*, 287, 193-197, 1980; Devos et al., *Nucleic Acids Res.* 10, 2487-2501, 1982; and Gray

et al., Nature, 295, 503-508, 1982). Alternatively, the desired DNA fragment can be obtained by screening a cDNA library prepared according to a known procedure (Okayama et al., Molecular and Cellular Biology, 3, 280, 1983)

5 and using a probe designed from a known nucleotide sequence described in the above-mentioned literature.

To obtain a DNA fragment coding for an interferon conjugate from cDNAs of interferons- $\beta$  and - $\gamma$ , the cDNAs are separately digested with an appropriate restriction  
10 enzyme, and the resulting cDNA fragments are ligated immediately or after blunt-ending with a mung bean nuclease, DNA polymerase I Klenow fragment or T4 DNA polymerase. The cDNA fragments are preferably linked via a synthetic oligonucleotide corresponding to  
15 nucleotides deleted by the restriction enzyme cleavage, to obtain a DNA fragment coding for an interferon conjugate comprising entire polypeptides of interferons- $\beta$  and - $\gamma$ . In such a case, according to one embodiment of the present invention, the synthetic oligonucleotide  
20 consists of a nucleotide sequence coding for a spacer peptide flanked by nucleotide sequences corresponding to nucleotides deleted by the restriction enzyme cleavage, so as to obtain a DNA fragment coding for an entire interferon conjugate comprising polypeptides of  
25 interferons- $\beta$  and - $\gamma$  linked via the spacer peptide. Alternatively, a restriction enzyme site is introduced in cDNAs of interferon- $\beta$  and interferon- $\gamma$  at their 5'-end or 3'-end of the structural gene using synthetic oligonucleotides, and the cDNAs are cleaved with an  
30 appropriate restriction enzyme, and if necessary, after the cDNAs are blunt-ended, the cDNAs are ligated. In any case, one cDNA fragment should be linked in reading frame with another cDNA fragment.

To produce an interferon conjugate using the  
35 above-mentioned DNA construct, animal or plant cells, yeast or E. coli are used as a host. To express an interferon conjugate, the DNA construct should have a

promoter for starting the transcription, and an SD sequence and ATG codon for starting the translation upstream therefrom. As a promoter, any nucleotide sequence having a promoter activity can be used; for example, lac promoter, trp promoter, recA promoter, and the like are known. Preferably a strong promoter such as trp promoter is used. The SD sequence is a ribosome RNA binding site and, therefore, essential for translation. In the present invention, any SD sequence can be used. A control region comprising the above-mentioned promoter and SD sequence thus constructed is added to a DNA fragment coding for an interferon conjugate via an ATG codon to express the interferon conjugate. The addition of the ATG codon can be carried out using synthetic oligonucleotides (Goeddel et al., Nature, 281, 544-548, 1979). Alternatively, for interferon- $\beta$ , the ATG codon can be exposed according to a known procedure (Taniguchi et al., Proc. Natl. Acad. Sci. USA, 77, 5230-5233, 1980).

To introduce the thus-obtained DNA into host cells, a vector DNA is used. Vector DNAs used for an E. coli host include a plasmid DNA such as pBR322 and pSC101, and a phage DNA such as  $\lambda$  phage. Such a DNA vector is linked with the DNA construct for expression of an interferon conjugate to construct an expression DNA vector, which is then used to transform host cells according to a known procedure (Maniatis et al., "Molecular Cloning", Cold Spring Harbor Laboratory, p250-255, 1982).

A transformant such as transformed E. coli is then cultured in an appropriate medium to produce an interferon conjugate. Culturing is preferably carried out in a liquid medium. When trp promoter is used, indoleacrylic acid is added to a culture medium during culturing to induce the production of an interferon conjugate. If a different promoter is used, an inducer selected in accordance with the particular promoter is

preferably used, to enhance the production of an interferon conjugate.

5 The interferon conjugate producer such as the thus-cultured E. coli cells are then disrupted according to a conventional cell disruption procedure ("Tanpakushitsu-Koso no Kisojikken" (Fundamental Experiment of Protein and Enzyme), edited by Takekazu Horio and Jinpei Yamashita, Nankodo, 1981, 3-7), for example, by treatment with a enzyme, sonication,  
10 grinding, or treatment under pressure, to obtain a crude extract containing an interferon conjugate.

The above-mentioned cell disruption procedure can be combined with treatment with guanidine hydrochloride or urea to improve the extraction efficiency (Davis et  
15 al., Gene, 21, 273-284, 1983).

The thus-obtained crude extract can be further purified according to a known procedure ("Tanpakushitsu-Koso no Kisojikken" (Fundamental Experiment of Protein and Enzyme), edited by Takekazu  
20 Horio and Jinpei Yamashita, Nankodo, 1981, 18-382), for example, by salting out, ultrafiltration, ion exchange, gel filtration, affinity chromatography, electrophoresis, or a combination thereof, to prepare a highly purified interferon conjugate preparation.

25 To express an interferon conjugate in animal cells, it is necessary that a nucleotide sequence coding for the interferon conjugate be present under the control of a promoter effective in the animal cells. Promoters effective in animal cells include, for example, SV40  
30 early promoter, SV40 late promoter, a promoter of a HB virus gene, MMTV promoter, a promoter of a thymidine kinase gene, a promoter of a heat shock protein, and a promoter of an interferon gene. A nucleotide sequence coding for an interferon conjugate is linked downstream  
35 of one of the above-mentioned promoters in the same manner as described for the expression in E. coli. A single promoter or a combination of more than one

promoter can be used. Note, an enhancer sequence of Harbey mouse sarcoma virus 5'LTR or an enhancer sequence of SV40 can be linked upstream of the above-mentioned promoter for a eukaryotic cell. Such enhancers are  
5 believed to increase the transcription efficiency. Preferably, a nucleotide sequence coding for signal peptide for extracellular secretion is added just upstream of the nucleotide sequence coding for an interferon conjugate, extracellularly to secrete the  
10 interferon conjugate, which can be then recovered in a supernatant of culture broth.

To prepare a large amount of the above-mentioned DNA construct so that the DNA construct can be easily introduced into animal cells, the DNA construct  
15 preferably includes an E. coli replication origin and a drug resistance gene. The replication origin is preferably, but not limited to, that derived from a colicin E1 plasmid, for example, pBR22, or a related plasmid. The drug resistance gene is, for example, an  
20 ampicillin resistant gene, tetracycline resistant gene, or kanamycin resistant gene. Moreover, the DNA construct can contain a replication origin responsible for autonomous replication in host cells, for example, a replication origin of SV40 virus or a replication origin  
25 of polyoma virus. These various DNA fragments are linked to construct an interferon conjugate expression vector.

The construction of a vector DNA can be carried out according to a conventional procedure (T. Maniatis et  
30 al., "Molecular Cloning", p86-96, 1982).

Animal cells into which a vector DNA will be introduced may be cells derived from humans, monkeys, Chinese hamsters, mice, etc., although since a human interferon conjugate is the target compound, human cells  
35 are preferably used. Human cells wherein growth is not inhibited by a peptide having glycoside chains produced are used. The human cells are preferably cells derived

from human lung cancer, especially PC8 and PC12 cell lines (M. Kinjo et al., Br. J. Cancer, 39, 15, 1979).

Vector DNA can be introduced into host cells according to a known calcium phosphate method (F.L.

5 Graham et al., Virology, 54, 536, 1973).

Cells into which a plasmid for expression of an interferon conjugate has been introduced can be efficiently obtained by cotransformation of host cells with the plasmid and G418 resistance gene expression  
10 vector pSV2neo (P.J. Southern et al., J. Mol. Appl. Genet., 1, 327, 1982) or pNE05' (M. Lusky et al., Cell, 36, 391, 1984), because the cotransformed cells can survive in a medium containing G418, in which non-cotransformed cells cannot survive.

15 The thus-obtained transformant is cultured in, for example, a medium containing fetal calf serum, extracellularly to produce an interferon conjugate, which is then recovered in a pure form from a supernatant of the cultured broth according to the same procedure as  
20 described for recovery from the E. coli extract. The thus-obtained interferon conjugate is a polypeptide having glycoside chains.

Since the interferon conjugate prepared as described above binds to an anti-human interferon- $\beta$  antibody and  
25 to an anti-human interferon- $\gamma$  antibody, it has the antigenecities of both interferons- $\beta$  and - $\gamma$ . Moreover, a neutralization test thereof with anti-human interferon- $\beta$  antibody and anti-human interferon- $\gamma$  antibody showed that a single polypeptide exhibits both  
30 interferon- $\beta$  activity and interferon- $\gamma$  activity.

As described above, the present invention provides a new type of interferon conjugate comprising a region exhibiting biological activities of interferon- $\beta$  and a region exhibiting biological activities of interferon- $\gamma$ ,  
35 which is produced by recombinant cells containing DNA coding for interferon- $\beta$  and interferon- $\gamma$ .

The interferon conjugate of the present invention

is a single polypeptide carrying biological actions which were originally carried separately on interferon- $\beta$  and interferon- $\gamma$ . Therefore, the present interferon conjugate has a broader action spectra, such as an antiviral spectrum and an anti-cell proliferation spectrum broader than those of conventional interferon. These properties make the present interferon conjugate valuable as an antiviral pharmaceutical or an antitumor pharmaceutical.

Moreover, since the present interferon conjugate exhibits the synergism conventionally provided by a mixture of interferon- $\beta$  and interferon- $\gamma$ , it provides remarkably enhanced activity per molecule in comparison to conventional interferons. Although such synergism can be obtained in vitro by only mixing interferon- $\beta$  and - $\gamma$ , since interferons- $\beta$  and - $\gamma$ , would show different in vivo pharmacodynamics, the coexistence of both interferons at a target site is not assured in vivo. This means that the mixture of both interferons- $\beta$  and - $\gamma$  would not exhibit the synergism. On the contrary, in the present interferon conjugate, since a single molecule provides the synergism, the problem of different pharmacodynamics does not exist. This also makes the present interferon conjugate valuable as an antiviral pharmaceutical and an antitumor pharmaceutical.

Finally, at present, to obtain interferon- $\beta$  activity and interferon- $\gamma$  activity, separate processes for the production of interferons- $\beta$  and - $\gamma$  must be carried out. On the contrary, according to the present invention, one production process provides an interferon conjugate carrying both interferon- $\beta$  activity and interferon- $\gamma$  activity. This gives an economical advantage to the present invention. The present interferon conjugate can be used as such, or can be easily cleaved into interferon- $\beta$  and interferon- $\gamma$ , which then can be used separately or as a mixture. In all cases the above-mentioned advantage still remains.



Examples

The present invention will now be further illustrated by, but is by no means limited to, the following examples.

5 Common procedures for gene manipulation used in the following Reference example and Examples are those described in Maniatis et al., "Molecular Cloning", Cold Spring Harbor Laboratory, 1982.

10 Prior to illustrating Examples of the present invention, the construction processes of a plasmid for the expression of human interferon- $\beta$  and a plasmid for the expression of human interferon- $\gamma$  are described as a Reference example. These plasmids are used as starting plasmids for the construction of the present plasmids.

15 Reference example

(1) Construction of plasmid pKM6 for expression of human interferon- $\beta$ .

A plasmid pTuIFN $\beta$ -5 for the expression of human interferon- $\beta$  constructed according to a reported procedure (Taniguchi, Seikagaku, 54, 363-377, 1982) was digested with HindIII, and the digestion product was treated with a T4 DNA polymerase Klenow fragment to make blunt ends, linked to a BglII linker, digested with BglII, and self-circularized using a T4 DNA ligase to obtain a plasmid pYO-10. The plasmid pYO-10 was digested with SalI and ClaI, and the digestion product was subjected to agarose gel electrophoresis to isolate a DNA fragment of about 830 bp. This DNA fragment was inserted into a ClaI-SalI region of the plasmid p6huy-A2 described in Japanese Unexamined Patent Publication No. 61-19488 to construct a plasmid pKM6 shown in Fig. 3.

(2) Construction of plasmid p6huy-N1 for expression of human interferon  $\gamma$ .

35 Lymphocyte derived from human tonsils was treated with phytohemagglutinin (PHA) and 12-o-tetradecanoyl-phorbol-13-acetate (TPA) to induce the production of human interferon- $\gamma$  (Vilvek et al.,

Infection and Immunity, 34, 131, 1983), and then mRNA was prepared from the treated cells. The preparations of mRNA and cDNA, and the cloning of cDNA in a plasmid were carried out according to known procedures (Okayama  
5 et al., Molecular and Cellular Biology, 3, 280, 1983). The DNA library thus obtained was subjected to colony hybridization using a synthetic oligonucleotide probe having the sequence: 5'-AGGACAACCATTACT-3' corresponding to a region near the 3'-terminal of a known human  
10 interferon- $\gamma$  structural gene (Goeddel et al., Nature, 295, 503-509, 1982) to obtain a plasmid pIFN- $\gamma$ 15 containing a cDNA coding for human interferon- $\gamma$ . Next, after the plasmid pIFN- $\gamma$ 15 was digested with NdeI, and BamHI, a DNA fragment of about 0.9 kb was isolated by  
15 agarose gel electrophoresis.

On the other hand, two oligonucleotides 5'-CGATGCAGGACCCA-3' and 5'-TATGGGTCCTGCAT-3' were synthesized, and after phosphorylation at the 5'-end with a T4 polynucleotide kinase, the oligonucleotides  
20 were mixed to make a concentration of about 8 pmol/ $\mu$ l for each oligonucleotide. The mixture was then heated at 65°C for 3 minutes, cooled rapidly, heated again at 65°C for 3 minutes, and then allowed to stand at a room temperature to be cooled gradually and to anneal the  
25 oligonucleotides.

7 pmoles of this oligonucleotide, 0.3 pmole of NdeI-BamHI fragment from plasmid pIFN $\gamma$ -15, and 0.1 pmole of a DNA fragment of about 4200 bp prepared by digestion of the plasmid pKM6 described in paragraph (1) with ClaI  
30 and BamHI followed by isolation by agarose gel electrophoresis were mixed, and after ligation the ligation mixture was used to transform E. coli MC1061 (Casadaban et al., J. Mol. Biol., 138, 179-207, 1980). The transformants selected for ampicillin resistance were sub-  
35 jected to colony hybridization using an oligonucleotide probe 5'-TATGGGTCCTGCAT-3', to obtain a plasmid p6hur-N1 for expression of human interferon- $\gamma$  (Fig. 4).

Next, to link the interferon- $\beta$  structural gene and interferon- $\gamma$  structural gene, restriction enzyme sites are introduced to the 5'-end or 3'-end of each structural gene.

5       (3) Construction of plasmid pKM6-Cxho

The structure of the plasmid pKM6-Cxho is shown in Fig. 5. The plasmid pKM6 constructed as described in paragraph (1) was digested with BstEII and BamHI, and an adapter oligonucleotide having the  
10 following sequence:

GTTACCTCCGAAACTCGAGCTGA

GAGGCTTTGAGCTCGACTCTAG

was linked to the cleaved plasmid to construct the plasmid pKM6-cxho. The adapter was prepared according  
15 to the same procedure as described in paragraph (2). When the plasmid pKM6-Cxho is digested with XhoI and the protruding nucleotides are eliminated, a codon AAC coding for aspartic acid present at the C-terminal of human interferon- $\beta$  is exposed.

20       (4) Construction of plasmid p6huyN1-CKpn

The structure of the plasmid p6huyN1-CKpn is shown in Fig. 6. The plasmid p6huy-N1 constructed as described in paragraph (2) was digested with ClaI and BamHI, and after agarose gel electrophoresis, a DNA  
25 fragment of about 4200 bp and a DNA fragment of about 1050 bp were isolated. The DNA fragment of about 1050 bp was further digested with HinfI, and after agarose gel electrophoresis a DNA fragment of about 400 bp was isolated. The ClaI-BamHI fragment of about  
30 4200 bp prepared as described above, the ClaI-HinfI fragment of about 400 bp, and an adapter of the following sequence:

(5')-AGTCAGATGCTGTTTCGGGTCGAGTCATCCCAGGTACCATGAGATCTG

GTCTACGACAAAGCGGCGAGCTGCAAGTAGGGTCCATGGTACTCTAGAOCTAG-(5')

35 were mixed and ligated, and the ligation mixture was used to transform E. coli MC1061. The above-mentioned adapter was prepared from six oligonucleotides according

to the same procedure as described in paragraph (2).  
The transformants selected for ampicillin resistance  
were subjected to colony hybridization using an  
oligonucleotide probe 5'-GATCCAGATCTCATG-3' to obtain  
5 four positive clones from 118 clones. The positive  
clones contained plasmid p6huγ-CKpn. When the thus-  
prepared plasmid p6huγ-CKpn is digested with KpnI, and  
protruding nucleotides are eliminated, a codon CAG  
coding for glutamine present at the C-terminal of human  
10 interferon-γ is exposed.

(5) Construction of plasmid p6huγN1ΔBS-NHin

The structure of plasmid p6huγN1ΔBS-NHin is  
shown in Fig. 7. The plasmid p6huγ-N1 constructed as  
described in paragraph (2) was digested with BstEII, the  
15 resulting cohesive ends were changed to blunt ends using  
a DNA polymerase I Klenow fragment, and the resulting DNA  
fragment was linked to a SalI linker. After digestion  
with SalI, the DNA fragment was self-circularized using  
the T4 DNA ligase to obtain p6huγN1-ΔBS. The plasmid  
20 p6huγN1-ΔBS was digested with NdeI and SalI, and after  
agarose gel electrophoresis, a DNA fragment of about  
3700 bp was isolated.

On the other hand, the plasmid pKM6 constructed  
as described in paragraph (2) was digested with EcoRI  
25 and SalI, and after agarose gel electrophoresis, a DNA  
fragment of about 3700 bp was isolated.

Two DNA fragments prepared as described above  
were linked with an adapter oligonucleotide of the  
following sequence:

AATTGCGCAGGACCCA

CGCGTCCTGGGTAT

to obtain plasmid p6huγN1ΔBS-NHin. When the plasmid  
p6huγN1ΔBS-NHin is digested with HinPI, and protruding  
nucleotides are eliminated, a codon CAG coding for  
35 glutamine present at the N-terminal of human interferon-γ  
is exposed.

Example 1. Construction of plasmid ptrp6huIFN-γ8

for expression of interferon- $\gamma$  $\cdot$  $\beta$   
conjugate

A process for the construction of a plasmid ptrp6huIFN- $\gamma$  $\beta$  is shown in Fig. 8, wherein 30  $\mu$ g of  
5 plasmid pKM6 was digested with ClaI, and the digestion product was treated with 15 units of mung bean nuclease to change cohesive ends to blunt ends. The DNA fragment thus-obtained was further digested with BglII, and after  
10 agarose gel electrophoresis, a DNA fragment of about 500 bp was isolated. On the other hand, plasmid p6hu $\gamma$ N1-CKpn was digested with KpnI, the cohesive ends were changed to blunt ends using T4 DNA polymerase, and after digestion with BamHI followed by agarose gel  
15 electrophoresis, a DNA fragment of about 4800 bp was obtained. The DNA fragments thus-prepared were mixed and ligated using T4 DNA ligase, and the reaction mixture was used to transform E. coli HB101 (Boyer et al., J. Mol. Biol., 41, 459-472, 1969). The resulting  
20 ampicillin resistant transformants were subjected to colony hybridization using as a probe a  $^{32}$ P-labeled DNA fragment prepared by nick-translating a ClaI-BglII fragment of plasmid pKM6, to obtain 6 positive clones among 56 clones. Plasmid DNA was extracted from each of  
25 these clones, and a restriction enzyme cleavage map was made. As a result, it was confirmed that plasmids from all positive clones had a structure as shown in Fig. 8. Moreover, plasmid DNA derived from a representative  
30 clone  $\gamma$  $\beta$ 6 was digested with SalI, and resulting DNA fragments were inserted to M13 phage to determine a nucleotide sequence of the plasmid DNA. As a result, in  
this plasmid, an IFN- $\gamma$  structural gene was linked with an IFN- $\beta$  structural gene in frame. This desired plasmid was designated as plasmid ptrp6huIFN- $\gamma$  $\beta$ , and a trans-  
35 formant E. coli was designated as E. coli HB101 (ptrp6huIFN- $\gamma$  $\beta$ ).

Example 2. Construction of plasmid ptrp6huIFN- $\beta$  $\alpha$   
for expression of interferon- $\beta$  $\cdot$  $\alpha$

conjugate

A process for the construction of a plasmid ptrp6huIFN- $\beta$  $\gamma$  is shown in Fig. 9, wherein 20  $\mu$ g of plasmid pKM6-Cxho was digested with Xho I, and the digestion product was treated with 15 units of mung bean nuclease at 37°C for 15 minutes to make blunt ends, and then digested with SalI. The reaction mixture was subjected to agarose gel electrophoresis to isolate a DNA fragment of about 4500 bp. On the other hand, 30  $\mu$ g of p6hu $\gamma$ N1 $\Delta$ BS-NH $\alpha$ n was digested with HinPI, the digestion product was treated with 30 units of mung bean nuclease at 37°C for 15 minutes, and digested with SalI, and after agarose gel electrophoresis a DNA fragment of about 860 bp was isolated.

The DNA fragments thus-prepared were mixed and ligated using the T4 DNA ligase, and the reaction mixture was used to transform E. coli HB101. Among the resulting ampicillin resistant transformants, 50 clones were subjected to colony hybridization using a oligonucleotide probe 5'-AGTCAGATGCTGTTTC-3', which is the same oligonucleotide used for the construction of plasmid p6hu $\gamma$ N1-CKpn, to obtain 28 positive clones. A plasmid DNA was isolated from a representative clone  $\beta$  $\gamma$ 31, and a restriction enzyme cleavage map was made. As a result, a map as shown in Fig. 9 was obtained. Moreover, a BstEII-SalI fragment from the plasmid isolated from the clone  $\beta$  $\gamma$ 31 was cloned in phage M13 to determine the nucleotide sequence. As a result, in the plasmid, an IFN- $\beta$  structural gene was linked with an IFN- $\gamma$  structural gene in frame. This plasmid was designated as plasmid ptrp6huIFN- $\beta$  $\gamma$ , and E. coli transformed with the plasmid was designated as E. coli HB101 (ptrp6huIFN- $\beta$  $\gamma$ ).

Example 3. Construction of plasmid ptrphuIFN- $\gamma$ c $\beta$  for expression of interferon- $\gamma$ c $\beta$  conjugate

A process for the construction of a ptrphuIFN- $\gamma$ c $\beta$

is shown in Fig. 10. A plasmid pKM6 was digested with ClaI and then with BglII, and after agarose gel electrophoresis a DNA fragment of about 500 bp was isolated. On the other hand, a DNA fragment coding for a spacer peptide was prepared from four synthetic oligonucleotides according to the same procedure as described in paragraph (2) of the Reference Example. 10 pmoles of this DNA fragment was mixed with the ClaI-BglII fragment of pKM6 isolated as described above and the DNA fragment of about 4800 bp derived from plasmid p6huγN1-CKpn as described in Example 1, these DNA fragments were ligated using the T4 DNA ligase, and the reaction mixture was used to transform E. coli HB101. 82 clones of the resulting ampicillin resistant transformants were subjected to colony hybridization using the probe described in Example 1 to obtain three positive clones. Plasmid DNAs were isolated from the three clones, and restriction enzyme cleavage maps were made. As a result, only one clone contained a plasmid having a desired structure. This plasmid was designated as plasmid ptrp6huIFN-γc8, and E. coli transformed with this plasmid was designated as E. coli HB101 (ptrp6huIFN-γc8).

Example 4. Culturing and production of interferon conjugate

Transformants obtained in Examples 1 to 3 were inoculated to LB medium (1.0% bactotrypton, 0.5% yeast extract, 0.5% sodium chloride, 0.1% glucose, pH 7.2 with sodium hydroxide) supplemented with 100 μg/ml tryptophan, and 100 μg/ml ampicillin, and cultivation was carried out at 30°C for 8 hours. This culture was then inoculated to M9 medium (0.3% mono potassium phosphate, 0.6% disodium phosphate, 0.1% ammonium chloride, and 0.5% sodium chloride, and further, separately sterilized 1 μg/ml Vitamine B<sub>1</sub> and 0.1 mM magnesium sulfate) supplemented with 1.0% glucose and 1.0% casamino acids, in an amount of 10% by volume relating to a volume of

the M9 medium, and cultivation was continued. After about 10 hours, indoleacrylic acid was added to the final concentration of 10  $\mu$ g/ml, and cultivation was carried out for an additional 8 hours. During the cultivation, if necessary, 40% glucose aqueous solution was added to the culture medium to prevent depletion of the glucose. Moreover, during the cultivation, a 14% aqueous ammonia solution was added to the culture medium to maintain the pH value at 6.0 to 7.0.

10 After the cultivation, 2 ml of the cultured broth was centrifuged at 10,000X g for 4 minutes to collect cells. The cells were washed with physiological saline, and the washed cells were suspended in 1 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 3 mg of lysozyme, 15 2 mM EDTA, 30 mM sodium chloride, and 20% glycerol, and the suspension was allowed to stand in ice for 60 minutes. The freezing and thawing of the suspension were repeated three times to disrupt the cells, and the disrupted mixture was centrifuged at 30,000X g for 20 20 minutes to eliminate cell debris. A supernatant thus obtained was used as a test sample for measuring.

The antiviral activity of interferon was measured according to "Interferon no Kagaku" (Science of Interferon) edited by Shigeyasu Kobayashi, pl3-20, Kodan 25 Sha, 1985, by the CPE<sub>50</sub> inhibition method using the FL cells-sindbis virus system. As a standard for measurement of the activity, an IFN- $\gamma$  preparation was used which was produced by recombinant cells and calibrated using NIH natural IFN- $\gamma$  Gg23-901-530. For 30 reference, interferon crude extracts prepared according to the above-mentioned procedure from E. coli HB101 containing plasmid pKM6, which expresses human interferon- $\beta$ , and from E. coli HB101 containing plasmid p6hur-N1, which express human interferon- $\gamma$ , were tested 35 for their antiviral activity. The results are shown in Table 1.



Table 1

Strain	Antiviral Activity per Extract (U/ml)
<u>E. coli</u> HB101 (ptrp6huIFN- $\gamma$ $\beta$ )	$3.9 \times 10^4$
<u>E. coli</u> HB101 (ptrp6huIFN- $\beta$ $\gamma$ )	$1.6 \times 10^4$
<u>E. coli</u> HB101 (ptrp6huIFN- $\gamma$ c $\beta$ )	$7.7 \times 10^4$
<u>E. coli</u> HB101 (pKM6)	$3.1 \times 10^5$
<u>E. coli</u> HB101 (p6hu $\gamma$ -N1)	$4.1 \times 10^4$

As seen from Table 1, each preparation exhibited an  
20 antiviral activity characteristic for the expected  
interferon.

Example 5. Measurement of Molecular Weight

1 ml of cultured broth prepared according to the  
same procedure as described in Example 4 was centrifuged  
25 at 10,000X g for 4 minutes to collect cells. The cells  
were suspended in 500  $\mu$ l of 62.5 mM Tris-HCl buffer  
(pH 6.8) containing 5% 2-mercaptoethanol and 2% sodium  
dodecylsulfate, and the suspension was heated in a  
boiling water bath for 5 minutes and allowed to cool.  
30 Then, to the heat-treated suspension was added 50  $\mu$ l of  
62.5 mM Tris-HCl buffer (pH 6.8) containing 0.05% bromo  
phenol blue and 70% glycerol, to prepare a sample for  
electrophoresis. SDS-polyacrylamide gel electrophoresis  
was carried out according to Laemmli's method (Nature,  
35 227, 680, 1970). The gel concentration was 15%. As  
marker proteins, lysozyme having a molecular weight of  
14,400, trypsin inhibitor having a molecular weight of

21,500, carbonic anhydrase having a molecular weight of 31,000, ovalbumin having a molecular weight of 45,000, bovine serum albumin having a molecular weight of 66,200, and phosphorylase B having a molecular weight of 92,500 were used. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R250 to detect proteins. Another gel obtained by simultaneous electrophoresis was used to transport proteins on the gel to a nitrocellulose sheet. The nitrocellulose sheet was reacted with commercially available anti-human interferon- $\beta$  immunoglobulin or anti-human interferon- $\gamma$  immunoglobulin, and then reacted with peroxidase-labeled protein A to determine the position of the interferon conjugate. By comparing a result of the above-mentioned Western blotting with the positions of electrophoretically migrated marker proteins, it was found that IFN- $\gamma\beta$  and IFN- $\beta\gamma$  have a molecular weight of about 37,000, and IFN- $\gamma\epsilon\beta$  has a molecular weight of about 38,000. That is, it was confirmed that interferon conjugates of the present invention substantially consist of human interferon- $\beta$  having a molecular weight of about 20,000 and human interferon- $\gamma$  having a molecular weight of about 17,000. Note, about 1000 of a molecular weight in IFN- $\gamma\epsilon\beta$  is assigned to a spacer peptide contained in the IFN- $\gamma\epsilon\beta$ .

Example 6. Neutralization of product with antibody

A crude interferon extract from the E. coli HB101 (ptrp6huIFN- $\gamma\beta$ ) prepared according to the procedure described in Example 4 was diluted five-fold with an Eagle's minimum essential medium containing 5% calf serum and 10 mM Hepes (pH 7.3). To 1 ml of the interferon solution thus-prepared, 1 ml of anti-IFN- $\beta$  rabbit antiserum diluted 50-fold with the same medium (neutralization value 2700 U/ml) or 1 ml of anti-IFN- $\gamma$  rabbit antiserum diluted 50-fold with the same medium (neutralization value 2000 U/ml) was added, and after the mixture was incubated at 37°C for 30 minutes, the

antiviral activity was measured. For control, a reaction mixture wherein the antiserum was replaced by the medium, and a reaction mixture wherein 0.5 ml of the anti-IFN- $\beta$  rabbit antiserum solution and 0.5 ml of the anti-IFN- $\gamma$  rabbit antiserum solution were added were prepared, and the antiviral activity was measured in same manner. The results are set forth in Table 2.

Table 2

Antiserum	Antiviral Activity (U/ml)
Control (no antiserum)	$6.0 \times 10^3$
Anti-IFN- $\beta$ antiserum	$1.3 \times 10^3$
Anti-IFN- $\gamma$ antiserum	$1.7 \times 10^3$
Anti-IFN- $\beta$ antiserum + and -IFN- $\gamma$ antiserum	$8.1 \times 10$

As seen from Table 2, each of the anti-IFN- $\beta$  antiserum and the anti-IFN- $\gamma$  antiserum partially neutralized antiviral activity, and a combination of anti-IFN- $\beta$  antiserum and anti-IFN- $\gamma$  antiserum substantially completely neutralized all antiviral activity. This means that the present interferon- $\gamma\beta$  conjugate has both an interferon- $\beta$  action and an interferon- $\gamma$  action. Moreover, although  $2.7 \times 10^3$  U of anti-IFN- $\beta$  antiserum neutralized the IFN- $\beta$  activity with  $1.3 \times 10^3$  U of IFN- $\gamma$  activity remaining and  $2.0 \times 10^3$  U of anti-IFN- $\gamma$  antiserum neutralized the IFN- $\gamma$  activity with  $1.7 \times 10^3$  U of IFN- $\beta$  activity remaining, the not-neutralized control showed  $6.0 \times 10^3$  U activity. That is, antiviral activity of the control ( $6.0 \times 10^3$ ) is higher than total amount of remaining activities ( $1.3 \times 10^3$  U +  $1.7 \times 10^3$  U). This means that the present interferon- $\gamma\beta$  conjugate exhibits synergism of IFN- $\beta$

activity and IFN- $\gamma$  activity.

Example 7. Construction of plasmid ptrp6huIFN- $\beta$ cy  
for expression of interferon  $\beta$ cy

A process for the construction of a plasmid ptrp6huIFN- $\beta$ cy is shown in Fig. 12, wherein 20  $\mu$ g of plasmid pKM6-Cxho was digested with XhoI, the digestion product was treated with 15 units of mung bean nuclease at 37°C for 15 minutes to make blunt ends, and then digested with SalI. The reaction mixture was subjected to agarose gel electrophoresis to isolate a DNA fragment of about 4500 bp. On the other hand, 30  $\mu$ g of plasmid p6hu $\gamma$ N1 $\Delta$ BS-NHin was digested with HinPI and SalI, and after agarose gel electrophoresis, a DNA fragment of about 860 bp was isolated. The above-mentioned two DNA fragments were mixed with 10 pmoles of DNA fragment coding for a spacer peptide obtained according to the procedure as described in Example 3, these three DNA fragments were ligated with T4 DNA ligase, and the ligation mixture was used to transform E. coli HB101. The 204 ampicillin resistant transformants thus obtained were subjected to colony hybridization using as a probe that shown in Example 2 or an oligonucleotide 5'-CGTTACCGACTTAGCA-3' used for preparation of the DNA fragment coding for the spacer peptide. As a result, two clones were found to be positive, and plasmids from these clones were analyzed by restriction enzymes. One clone contained a desired plasmid, and this plasmid was designated as plasmid ptrp6huIFN- $\beta$ cy, and E. coli transformed with this plasmid was designated as E. coli HB101 (ptrp6huIFN- $\beta$ cy). According to the procedures described in Example 4, the E. coli transformant was cultured and an extract prepared, and then the antiviral activity was measured. An antiviral activity of  $3.9 \times 10^4$  U/ml extract was found.

35 Example 8. Neutralization of product with antibody

According to the same procedure as described in Example 6, a crude interferon extract from E. coli HB101

(ptrp6huIFN- $\gamma$ c $\beta$ ) was neutralized with antisera. For comparison, an interferon mixture consisting of 8600 U/ml of IFN- $\beta$  and 2400 U/ml of IFN- $\gamma$ , prepared by mixing an IFN- $\beta$  and an IFN- $\gamma$  in approximately the same molecular numbers, were tested in the same manner. The results are shown in Table 3.

Table 3

IFN	Antiserum		Antiviral activity (U/ml)
	Anti-IFN- $\beta$	Anti-IFN- $\gamma$	
IFN mixture	-	-	19000
	o	-	930
	-	o	12000
	o	o	<27
IFN- $\gamma$ c $\beta$	-	-	22000
	o	-	2400
	-	o	11000
	o	o	61

As can be seen from Table 3, in the interferon- $\gamma$ c $\beta$  conjugate (IFN- $\gamma$ c $\beta$ ), each of the anti-IFN- $\beta$  antiserum and anti-IFN- $\gamma$  antiserum partially neutralized the antiviral activity thereof, and a combination of both antisera substantially completely neutralized all antiviral activity. This means that correctly folded IFN- $\beta$  and IFN- $\gamma$  molecules are liked to yield both the IFN- $\beta$  activity and IFN- $\gamma$  activity are exhibited by a single polypeptide.

Moreover, from the comparison of the activities resulting from neutralization with the anti-IFN- $\beta$  or anti-IFN- $\gamma$  with the activity resulting from

neutrolization with of a combination of anti-IFN- $\beta$  and anti-IFN- $\gamma$ , it appears that the present IFN- $\gamma$ C $\beta$  conjugate also exhibits the synergism of the IFN- $\beta$  activity and the IFN- $\gamma$  activity.

5        Example 9.

A.    Construction of vector for expression of human interferon- $\beta$

         pSV $\beta$  is a vector derived from the human interferon- $\gamma$  expression vector pSV2IFN $\beta$  (Japanese  
10    Unexamined Patent Publication No. 61-52283) by deleting a sequence inhibiting replication of the vector in a eukaryotic cell (M. Losky et al., Nature, 293 77, 1981). The vector pSV $\beta$  was constructed as follows.

         First, a PvuII site positioned in the pSV2IFN $\beta$   
15    upstream from the SV40 early promoter was replaced by a SalI site using a SalI linker, and the modified vector was cleaved with SalI and BamHI to isolate a 1.7 Kb DNA fragment required for expression of the human interferon- $\beta$ .

20        Next, vector pML2d (M. Lusky et al., Nature, 293, 79, 1981) derived from pBR322 by deleting a sequence inhibiting replication of the vector in the eukaryotic cell was cleaved with SalI and BamHI to isolate a long DNA fragment.

25        These two DNA fragments were ligated using T4 DNA ligase to obtain pSV $\beta$ .

B.    Construction of vector pMTV $\beta$  for expression of human interferon- $\beta$

         The vector pSV $\beta$  constructed in Section A was  
30    cleaved with SalI, and the SalI site in the vector was replaced by a HindIII site using a HindIII linker, and the modified vector was cleaved with HindIII to isolate a 3.8 Kb DNA fragment which does not contain the SV40 early promoter. The DNA fragment thus obtained was then  
35    treated with E. coli alkaline phosphatase to eliminate a terminal phosphate group.

         Next, vector pMTVdhfr (F. Lee et al., Nature,

294, 228, 1982) containing MMTV promoter was cleaved with a restriction enzyme HindIII to isolate a 1.4 Kb DNA fragment containing MMTV promoter.

These two DNA fragments were ligated using T4 DNA ligase to obtain pMTV $\beta$ .

C. Construction of vector pMTV $\gamma$  for expression of human interferon- $\gamma$

pMTV $\gamma$  is a vector wherein a human interferon- $\gamma$  gene is positioned under control by an MMTV promoter.

The vector pMTV $\beta$  constructed in Section B was cleaved at a HindIII site downstream from the MMTV promoter and at the BglII site downstream from the human interferon gene, and after the resulting fragments were blunt-ended using a DNA polymerase I Klenow fragment, a 3.9 Kb DNA fragment containing the MMTV promoter was isolated.

This DNA fragment was ligated with a 0.8 Kb DNA fragment containing a human interferon- $\gamma$  gene, which was obtained from pSVIFN $\gamma$  (Japanese Unexamined Patent Publication No. 61-52286) by cleaving with DpnI, using a T4 DNA ligase to obtain pMTV $\gamma$ .

D. Construction of vector pMTV(SV) $\gamma$  for expression of human interferon- $\gamma$

pMTV(SV) $\gamma$  is a vector derived from pMTV $\gamma$  by inserting an SV40 early promoter upstream of the MMTV promoter. The vector pMTV(SV) $\gamma$  was constructed as follows.

pMTV $\gamma$  constructed in Section C was cleaved with SalI, and after the resulting DNA fragment was blunt-ended using a DNA polymerase I Klenow fragment, a terminal phosphate group was eliminated by BAP treatment.

Next, pSV2IFN $\beta$  (Japanese Unexamined Patent Publication 61-52283) was cleaved with PvuII and HindIII to isolate a 0.3 Kb DNA fragment containing the SV40 early promoter, and the fragment was blunt-ended by treating with a DNA polymerase I Klenow fragment.

These two DNA fragments were ligated using T4

DNA ligase to obtain PMTV(SV) $\gamma$ .

E. Construction of plasmid PMTV(SV) $\gamma$  $\cdot$  $\beta$  for  
expression of human interferon- $\gamma$  $\cdot$  $\beta$  conjugate  
in animal cells (Fig. 13)

5 PMTV(SV) $\gamma$  $\cdot$  $\beta$  is a vector wherein the human  
interferon- $\gamma$  gene in PMTV(SV) $\gamma$  has been replaced by the  
human interferon- $\gamma$  $\cdot$  $\beta$  conjugate gene, and was constructed  
as follows.

10 10  $\mu$ g of ptrp6huIFN- $\gamma$  $\beta$  constructed according  
to the procedure as described in Example 1 was digested  
with NdeI and DpnI, and the digestion mixture was  
subjected to agarose gel electrophoresis to isolate a  
DNA fragment of about 1300 bp.

15 On the other hand, PMTV(SV) $\gamma$  constructed in  
Section D was digested with BglII, and the resulting  
fragment was blunt-ended by treating with DNA  
polymerase I Klenow fragment, followed by digestion with  
NdeI, and the reaction mixture was subjected to agarose  
gel electrophoresis to isolate a DNA fragment of about  
20 5100 bp.

These DNA fragments were mixed and ligated  
with T4 DNA ligase to obtain PMTV(SV) $\gamma$  $\cdot$  $\beta$ .

Example 10. Construction of plasmid PMTV(SV) $\gamma$ c $\beta$   
for expression of human interferon- $\gamma$ c $\beta$   
conjugate in animal cells

25 Plasmid PMTV(SV) $\gamma$ c $\beta$  is a vector wherein a human  
interferon- $\gamma$  gene in PMTV(SV) $\gamma$  has been replaced by a  
human interferon- $\gamma$ c $\beta$  conjugate gene, and was constructed  
as follows.

30 10  $\mu$ g of ptrp6huIFN- $\gamma$ c $\beta$  constructed according to  
the procedure as described in Example 3 was digested  
with NdeI and DpnI, and the reaction mixture was  
subjected to agarose gel electrophoresis to isolate a  
DNA fragment of about 1300 bp. On the other hand,  
35 PMTV(SV) $\gamma$  constructed in Section D was digested with  
BglII, and the resulting DNA fragment was blunt-ended by  
treating with a DNA polymerase I Klenow fragment,



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followed by digestion with NdeI, and the reaction mixture was subjected to agarose gel electrophoresis to isolate a DNA fragment of about 5100 bp.

These DNA fragments were mixed, and ligated using  
5 T4 DNA ligase to obtain pMTV(SV) $\gamma$ c8.

Example 11. Transformation of PC12 cell with  
MTV(SV) $\gamma$ ·8

4  $\mu$ g of vector pMTV(SV) $\gamma$ ·8 constructed in Example 9 and 0.4  $\mu$ g of GCl8 resistance gene expression vector  
10 pSV2neo (J. Southern et al., J. Mo. Appl. Genet., 1, 327, 1982) were introduced into about  $10^6$  cells of PC12 cell line (M. Kinjo et al., Br. J. Cancer, 39, 15, 1979) derived from human lung cancer, according to the calcium phosphate method. The cells thus-prepared were cultured  
15 in a selective medium (RPMI 1640 medium supplemented with 10% fetal calf serum and 100  $\mu$ g/ml kanamycine; Nissui Seiyaku, Japan) containing 400  $\mu$ g/ml of a protein synthesis inhibitor, GCl8 (GIBCO) to obtain 24 transformant clones. The antiviral activity of the cultured  
20 supernatants was tested using FL cells-sindbis virus system according to the CPE<sub>50</sub> inhibition method described in Example 4. As a result, supernatants from 22 clones exhibited the antiviral activity, as shown in Table 4.

Table 4

pMTV(SV)γ-B/PC12	
Clone	Antiviral activity (U/ml)
1	18500
2	1100
3	600
4	1500
5	< 80
6	1300
7	200
8	2300
9	200
10	< 80
11	1000
12	2500
13	900
14	1400
15	500
16	400
17	< 80
18	< 80
19	300
20	800
21	200
22	300
23	200
24	600

Example 12. Transformation of PC12 cells with  
pMTV(SV) $\gamma$ cb

4  $\mu$ g of pMTV(SV) $\gamma$ cb constructed in Example 10 and  
0.4  $\mu$ g of pSV2neo (see Example 11) were introduced into  
5 about  $10^6$  cells of the PC12 cell line by a calcium  
phosphate method according to the same procedure as  
described in Example 11. The cells thus-prepared were  
cultured in a selective medium (RPMI 1640 medium  
supplemented with 10% fetal calf serum and 100  $\mu$ g/ml  
10 kanamycine; Nissui Seiyaku, Japan) to obtain 26  
transformed clones. The antiviral activity of the  
cultured supernatants was tested by the CPE<sub>50</sub> inhibition  
method using the FL cells-sindbis virus system according  
to the same procedure as described in Example 11. As a  
15 result, supernatants from 20 clones exhibited the  
antiviral activity as shown in Table 5.

Table 5

pMTV(SV) $\gamma$ c8/PC12	
Clone	Antiviral activity (U/ml)
1	400
2	< 60
3	< 60
4	3800
5	1200
6	< 60
7	6400
8	200
9	< 80
10	400
11	200
12	900
13	500
14	700
15	< 80
16	1300
17	21450
18	130
19	5300
20	1600
21	200
22	< 80
23	1200
24	8600
25	300
26	500

CLAIMS

1. An interferon conjugate comprising in a single molecule a region exhibiting biological activities of interferon- $\beta$  and a region exhibiting biological activities of interferon- $\gamma$ .
- 5 2. An interferon conjugate according to claim 1, which is a biologically active polypeptide having no glycoside chain.
3. An interferon conjugate according to claim 1, which is a biologically active polypeptide having  
10 glycoside chains.
4. An interferon conjugate according to any one of claims 1 to 3, wherein the region exhibiting biological activities of interferon- $\beta$  and the region exhibiting biological activities of interferon- $\gamma$  have  
15 been linked via a spacer.
5. An interferon conjugate according to claim 4, wherein the spacer is a polypeptide.
6. An interferon conjugate according to any one of claims 1 to 3, wherein a C-terminal of the region  
20 exhibiting biological activities of interferon- $\beta$  has been linked to an N-terminal of the region exhibiting biological activities of interferon- $\gamma$ , directly or via a spacer.
7. An interferon conjugate according to claim 6,  
25 wherein the interferon- $\beta$  consists of the following amino acid sequence:

MET SER TYR ASN LEU LEU GLY PHE LEU GLN ARG SER SER  
ASN PHE GLN CYS GLN LYS LEU LEU TRP GLN LEU ASN GLY ARG LEU GLU TYR  
CYS LEU LYS ASP ARG MET ASN PHE ASP ILE PRO GLU GLU ILE LYS GLN LEU  
GLN GLN PHE GLN LYS GLU ASP ALA ALA LEU THR ILE TYR GLU MET LEU GLN  
ASN ILE PHE ALA ILE PHE ARG GLN ASP SER SER SER THR GLY TRP ASN GLU  
THR ILE VAL GLU ASN LEU LEU ALA ASN VAL TYR HIS GLN ILE ASN HIS LEU  
LYS THR VAL LEU GLU GLU LYS LEU GLU LYS GLU ASP PHE THR ARG GLY LYS  
LEU MET SER SER LEU HIS LEU LYS ARG TYR TYR GLY ARG ILE LEU HIS TYR  
LEU LYS ALA LYS GLU TYR SER HIS CYS ALA TRP THR ILE VAL ARG VAL GLU  
ILE LEU ARG ASN PHE TYR PHE ILE ASN ARG LEU THR GLY TYR LEU ARG ASN,

and the interferon- $\gamma$  consists of the following amino acid sequence:

GLN ASP PRO TYR VAL LYS GLU ALA GLU ASN LEU LYS LYS  
TYR PHE ASN ALA GLY HIS SER ASP VAL ALA ASP ASN GLY THR LEU PHE LEU  
GLY ILE LEU LYS ASN TRP LYS GLU GLU SER ASP ARG LYS ILE MET GLN SER  
GLN ILE VAL SER PHE TYR PHE LYS LEU PHE LYS ASN PHE LYS ASP ASP GLN  
SER ILE GLN LYS SER VAL GLU THR ILE LYS GLU ASP MET ASN VAL LYS PHE  
PHE ASN SER ASN LYS LYS LYS ARG ASP ASP PHE GLU LYS LEU THR ASN TYR  
SER VAL THR ASP LEU ASN VAL GLN ARG LYS ALA ILE HIS GLU LEU ILE GLN  
VAL MET ALA GLU LEU SER PRO ALA ALA LYS THR GLY LYS ARG LYS ARG SER  
GLN MET LEU PHE ARG GLY ARG ARG ALA SER GLN.

8. An interferon conjugate according to claim 7, wherein a C-terminal of a polypeptide consisting of the following amino acid sequence:

MET SER TYR ASN LEU LEU GLY PHE LEU GLN ARG SER SER  
ASN PHE GLN CYS GLN LYS LEU LEU TRP GIN LEU ASN GLY ARG LEU GLU TYR  
CYS LEU LYS ASP ARG MET ASN PHE ASP ILE PRO GLU GLU ILE LYS GLN LEU  
GLN GLN PHE GLN LYS GLU ASP ALA ALA LEU THR ILE TYR GLU MET LEU GLN  
ASN ILE PHE ALA ILE PHE ARG GLN ASP SER SER SER THR GLY TRP ASN GLU  
THR ILE VAL GLU ASN LEU LEU ALA ASN VAL TYR HIS GLN ILE ASN HIS LEU  
LYS THR VAL LEU GLU GLU LYS LEU GLU LYS GLU ASP PHE THR ARG GLY LYS  
LEU MET SER SER LEU HIS LEU LYS ARG TYR TYR GLY ARG ILE LEU HIS TYR  
LEU LYS ALA LYS GLU TYR SER HIS CYS ALA TRP THR ILE VAL ARG VAL GLU  
ILE LEU ARG ASN PHE TYR PHE ILE ASN ARG LEU THR GLY TYR LEU ARG ASN  
has been linked to an N-terminal of a polypeptide consisting of the following amino acid sequence:

GLN ASP PRO TYR VAL LYS GLU ALA GLU ASN LEU LYS LYS  
TYR PHE ASN ALA GLY HIS SER ASP VAL ALA ASP ASN GLY THR LEU PHE LEU  
GLY ILE LEU LYS ASN TRP LYS GLU GLU SER ASP ARG LYS ILE MET GLN SER  
GLN ILE VAL SER PHE TYR PHE LYS LEU PHE LYS ASN PHE LYS ASP ASP GLN  
SER ILE GLN LYS SER VAL GLU THR ILE LYS GLU ASP MET ASN VAL LYS PHE  
PHE ASN SER ASN LYS LYS LYS ARG ASP ASP PHE GLU LYS LEU THR ASN TYR  
SER VAL THR ASP LEU ASN VAL GLN ARG LYS ALA ILE HIS GLU LEU ILE GLN  
VAL MET ALA GLU LEU SER PRO ALA ALA LYS THR GLY LYS ARG LYS ARG SER  
GLN MET LEU PHE ARG GLY ARG ARG ALA SER GLN,  
via a polypeptide as a spacer.

9. An interferon conjugate according to any one

of claims 1 to 3, wherein a C-terminal of the region exhibiting biological activities of interferon- $\gamma$  has been linked to an N-terminal of the region exhibiting biological activities of interferon- $\beta$ , directly or via a spacer.

10. An interferon conjugate according to claim 9, wherein the interferon- $\gamma$  consists of the following amino acid sequence:

GLN ASP PRO TYR VAL LYS GLU ALA GLU ASN LEU LYS LYS  
TYR PHE ASN ALA GLY HIS SER ASP VAL ALA ASP ASN GLY THR LEU PHE LEU  
GLY ILE LEU LYS ASN TRP LYS GLU GLU SER ASP ARG LYS ILE MET GLN SER  
GLN ILE VAL SER PHE TYR PHE LYS LEU PHE LYS ASN PHE LYS ASP ASP GLN  
SER ILE GLN LYS SER VAL GLU THR ILE LYS GLU ASP MET ASN VAL LYS PHE  
PHE ASN SER ASN LYS LYS LYS ARG ASP ASP PHE GLU LYS LEU THR ASN TYR  
SER VAL THR ASP LEU ASN VAL GLN ARG LYS ALA ILE HIS GLU LEU ILE GLN  
VAL MET ALA GLU LEU SER PRO ALA ALA LYS THR GLY LYS ARG LYS ARG SER  
GLN MET LEU PHE ARG GLY ARG ARG ALA SER GLN,

and the interferon- $\beta$  consists of the following amino acid sequence:

MET SER TYR ASN LEU LEU GLY PHE LEU GLN ARG SER SER  
ASN PHE GLN CYS GLN LYS LEU LEU TRP GLN LEU ASN GLY ARG LEU GLU TYR  
CYS LEU LYS ASP ARG MET ASN PHE ASP ILE PRO GLU GLU ILE LYS GLN LEU  
GLN GLN PHE GLN LYS GLU ASP ALA ALA LEU THR ILE TYR GLU MET LEU GLN  
ASN ILE PHE ALA ILE PHE ARG GLN ASP SER SER SER THR GLY TRP ASN GLU  
THR ILE VAL GLU ASN LEU LEU ALA ASN VAL TYR HIS GLN ILE ASN HIS LEU  
LYS THR VAL LEU GLU GLU LYS LEU GLU LYS GLU ASP PHE THR ARG GLY LYS  
LEU MET SER SER LEU HIS LEU LYS ARG TYR TYR GLY ARG ILE LEU HIS TYR  
LEU LYS ALA LYS GLU TYR SER HIS CYS ALA TRP THR ILE VAL ARG VAL GLU  
ILE LEU ARG ASN PHE TYR PHE ILE ASN ARG LEU THR GLY TYR LEU ARG ASN.

30. 11. An interferon conjugate according to claim 10, wherein a C-terminal of a polypeptide consisting of the following amino acid sequence:

GLN ASP PRO TYR VAL LYS GLU ALA GLU ASN LEU LYS LYS  
TYR PHE ASN ALA GLY HIS SER ASP VAL ALA ASP ASN GLY THR LEU PHE LEU  
GLY ILE LEU LYS ASN TRP LYS GLU GLU SER ASP ARG LYS ILE MET GLN SER  
GLN ILE VAL SER PHE TYR PHE LYS LEU PHE LYS ASN PHE LYS ASP ASP GLN  
SER ILE GLN LYS SER VAL GLU THR ILE LYS GLU ASP MET ASN VAL LYS PHE

PHE ASN SER ASN LYS LYS LYS ARG ASP ASP PHE GLU LYS LEU THR ASN TYR  
 SER VAL THR ASP LEU ASN VAL GLN ARG LYS ALA ILE HIS GLU LEU ILE GLN  
 VAL MET ALA GLU LEU SER PRO ALA ALA LYS THR GLY LYS ARG LYS ARG SER  
 GLN MET LEU PHE ARG GLY ARG ARG ALA SER GLN

has been linked to an N-terminal of a polypeptide  
 consisting of the following amino acid sequence:

MET SER TYR ASN LEU LEU GLY PHE LEU GLN ARG SER SER  
 ASN PHE GLN CYS GLN LYS LEU LEU TRP GLN LEU ASN GLY ARG LEU GLU TYR  
 CYS LEU LYS ASP ARG MET ASN PHE ASP ILE PRO GLU GLU ILE LYS GLN LEU  
 GLN GLN PHE GLN LYS GLU ASP ALA ALA LEU THR ILE TYR GLU MET LEU GLN  
 ASN ILE PHE ALA ILE PHE ARG GLN ASP SER SER SER THR GLY TRP ASN GLU  
 THR ILE VAL GLU ASN LEU LEU ALA ASN VAL TYR HIS GLN ILE ASN HIS LEU  
 LYS THR VAL LEU GLU GLU LYS LEU GLU LYS GLU ASP PHE THR ARG GLY LYS  
 LEU MET SER SER LEU HIS LEU LYS ARG TYR TYR GLY ARG ILE LEU HIS TYR  
 LEU LYS ALA LYS GLU TYR SER HIS CYS ALA TRP THR ILE VAL ARG VAL GLU  
 ILE LEU ARG ASN PHE TYR PHE ILE ASN ARG LEU THR GLY TYR LEU ARG ASN,  
 via a polypeptide as a spacer

12. A deoxyribonucleotide sequence coding for an  
 interferon conjugate comprising in a single molecule a  
 20 region exhibiting biological activities of interferon- $\beta$   
 and a region exhibiting biological activities of  
 interferon- $\gamma$ .

13. A recombinant DNA comprising a deoxyribon-  
 ucleotide sequence coding for an interferon conjugate  
 25 comprising in a single molecule a region exhibiting  
 biological activities of interferon- $\beta$  and a region  
 exhibiting biological activities of interferon- $\gamma$ , and a  
 deoxyribonucleotide sequence coding for a control region  
 for expression of said interferon conjugate, wherein the  
 30 latter sequence is upstream from the former sequence.

14. A transformant organism transformed with a  
 recombinant DNA comprising a deoxyribonucleotide sequence  
 coding for an interferon conjugate comprising in a  
 single molecule a region exhibiting biological activities  
 35 of interferon- $\beta$  and a region exhibiting biological  
 activities of interferon- $\gamma$ , and a deoxyribonucleotide  
 sequence coding for a control region for expression of



said interferon conjugate, wherein the latter sequence is upstream from the former sequence.

15. A process for production of an interferon conjugate comprising in a single molecule a region  
5 exhibiting biological activities of interferon- $\beta$  and a region exhibiting biological activities of interferon- $\beta$ , comprising the step:

10 culturing a transformant organism transformed with a recombinant DNA comprising a deoxyribonucleotide sequence coding for said interferon conjugate and a deoxyribonucleotide sequence coding for a control region for expression of the interferon conjugate wherein the latter sequence is upstream from the former sequence, to produce the interferon conjugate;

- 15 and recovering said interferon conjugate.

16. A process according to claim 15, wherein E. coli is used as a host.

17. A process according to claim 11, wherein human cells are used as a host.

- 20 18. A process according to claim 17, wherein the human cells are cells derived from human lung cancer.

19. A process according to claim 18, wherein the cells derived from human lung cancer are selected from the group consisting of PC8 and PC12 cell lines.

# Fig. 1

MET SER TYR ASN

LEU LEU GLY PHE LEU GIN ARG SER SER ASN PHE GIN CYS GIN LYS LEU LEU TRP GIN LEU ASN GLY ARG LEU GLU

TYR CYS LEU LYS ASP ARG MET ASN PHE ASP ILE PRO GLU GLU ILE LYS GIN LEU GIN PHE GIN LYS GLU ASP

ALA ALA LEU THR ILE TYR GLU MET LEU GIN ASN ILE PHE ALA ILE PHE ARG GIN ASP SER SER THR GLY TRP

ASN GLU THR ILE VAL GLU ASN LEU LEU ALA ASN VAL TYR HIS GIN ILE ASN HIS LEU LYS THR VAL LEU GLU GLU

LYS LEU GLU LYS GLU ASP PHE THR ARG GLY LYS LEU MET SER SER LEU HIS LEU LYS ARG TYR TYR GLY ARG ILE

LEU HIS TYR LEU LYS ALA LYS GLU TYR SER HIS CYS ALA TRP THR ILE VAL ARG VAL GLU ILE LEU ARG ASN PHE

TYR PHE ILE ASN ARG LEU THR GLY TYR LEU ARG ASN

## Fig. 2

GLY ASP PRO TYR VAL LYS GLU ALA GLU ASN LEU LYS LYS TYR PHE ASN ALA GLY HIS SER ASP VAL

ALA ASP ASN GLY THR LEU PHE LEU GLY ILE LEU LYS ASN TRP LYS GLU GLU SER ASP ARG LYS ILE MET GIN SER

GLN ILE VAL SER PHE TYR PHE LYS LEU PHE LYS ASN PHE LYS ASP ASP GIN SER ILE GIN LYS SER VAL GLU THR

ILE LYS GLU ASP MET ASN VAL LYS PHE PHE ASN SER ASN LYS LYS LYS ARG ASP PHE GLU LYS LEU THR ASN

TYR SER VAL THR ASP LEU ASN VAL GIN ARG LYS ALA ILE HIS GLU LEU ILE GIN VAL MET ALA GLU LEU SER PRO

ALA ALA LYS THR GLY LYS ARG LYS ARG SER GIN MET LEU PHE ARG GLY ARG ARG ALA SER GIN

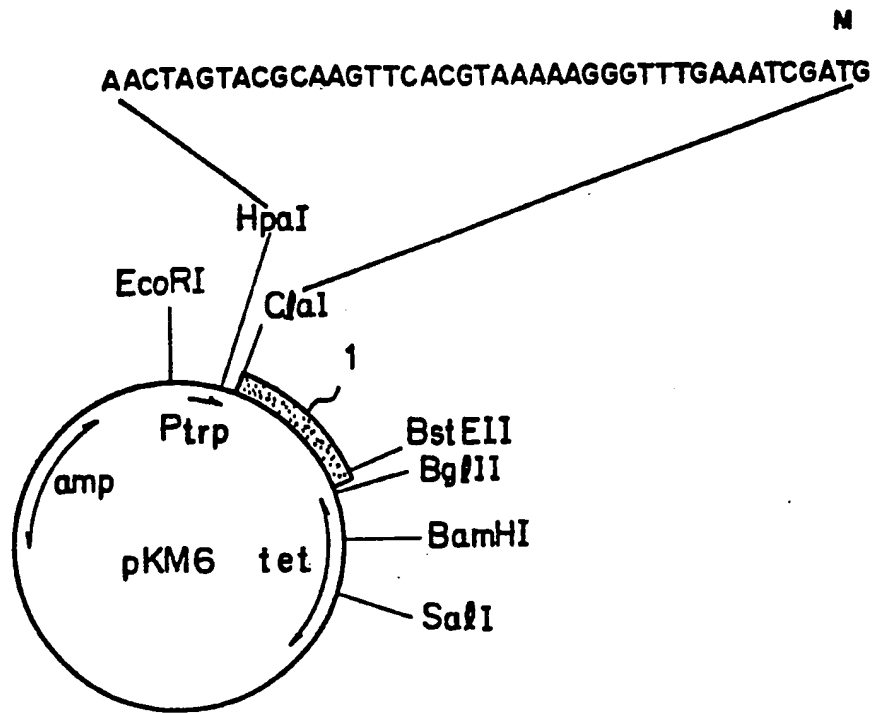
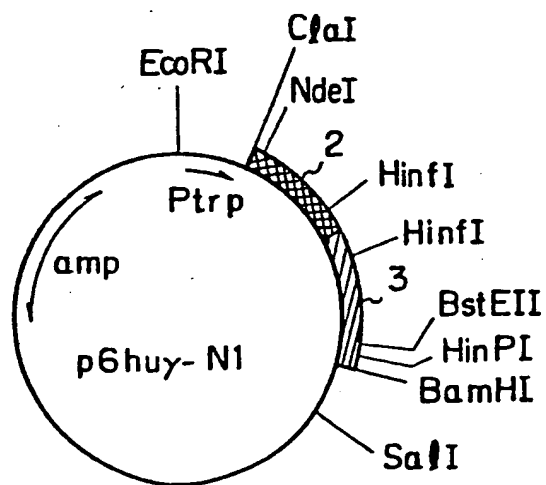
*Fig. 3**Fig. 4*

Fig. 5

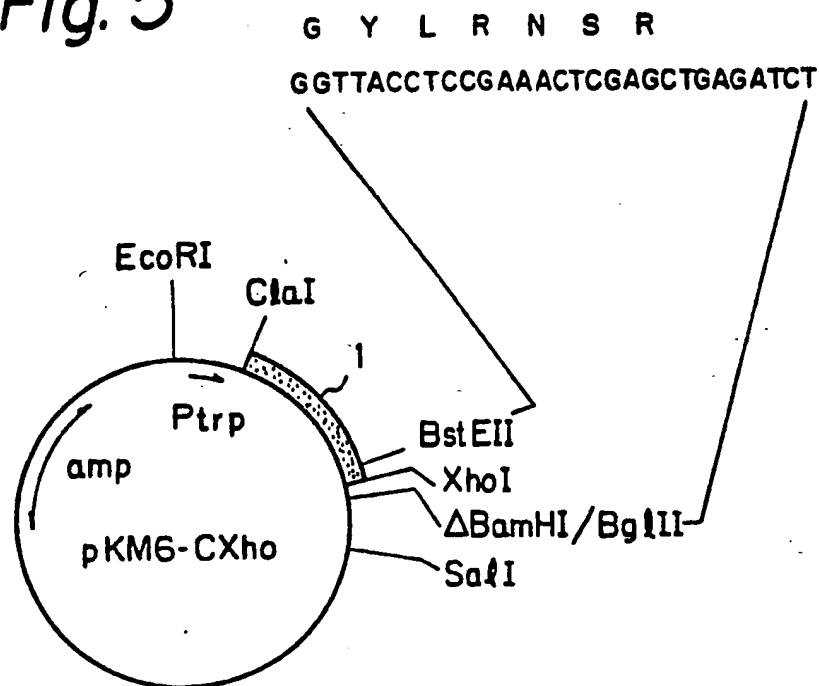


Fig. 6

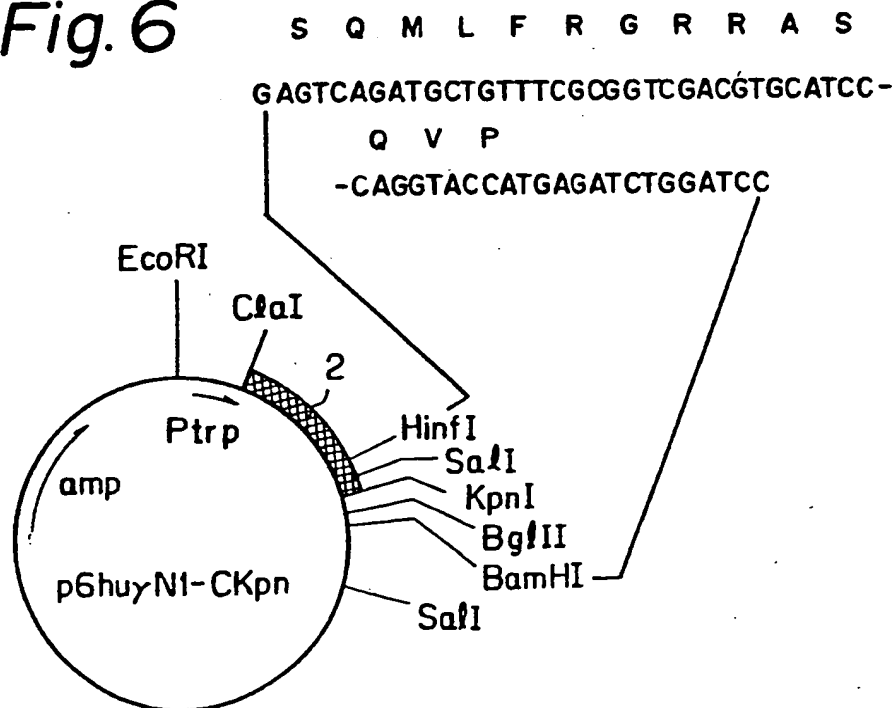


Fig. 7

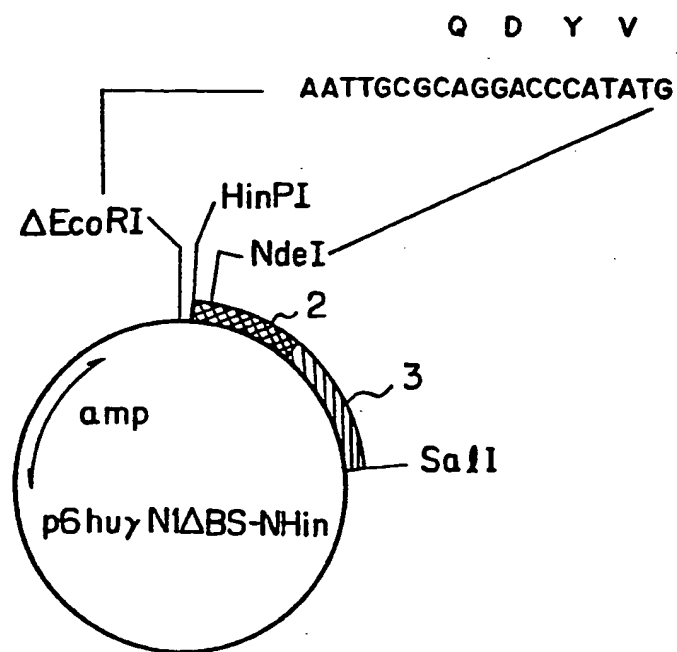


Fig. 8

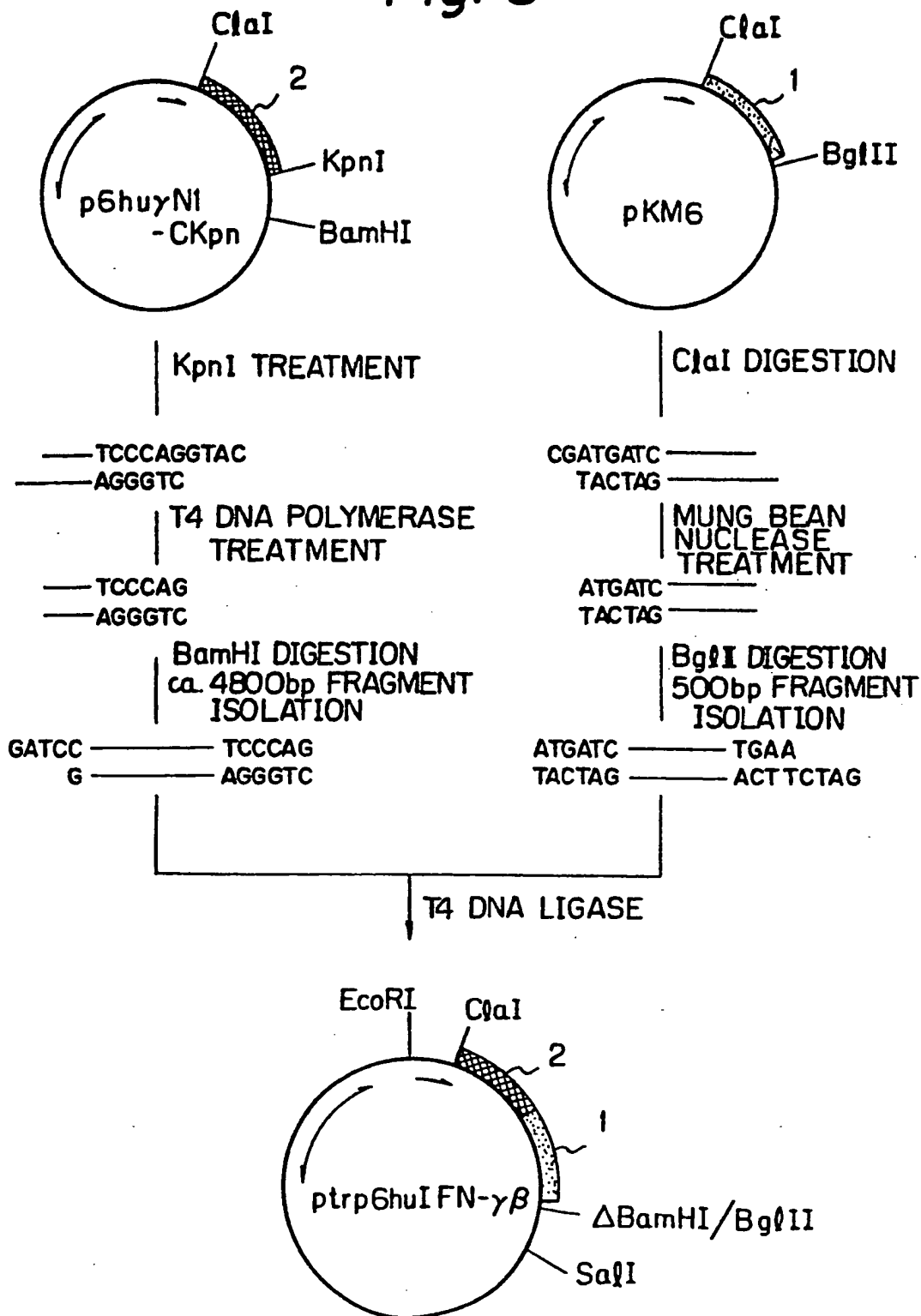


Fig. 9

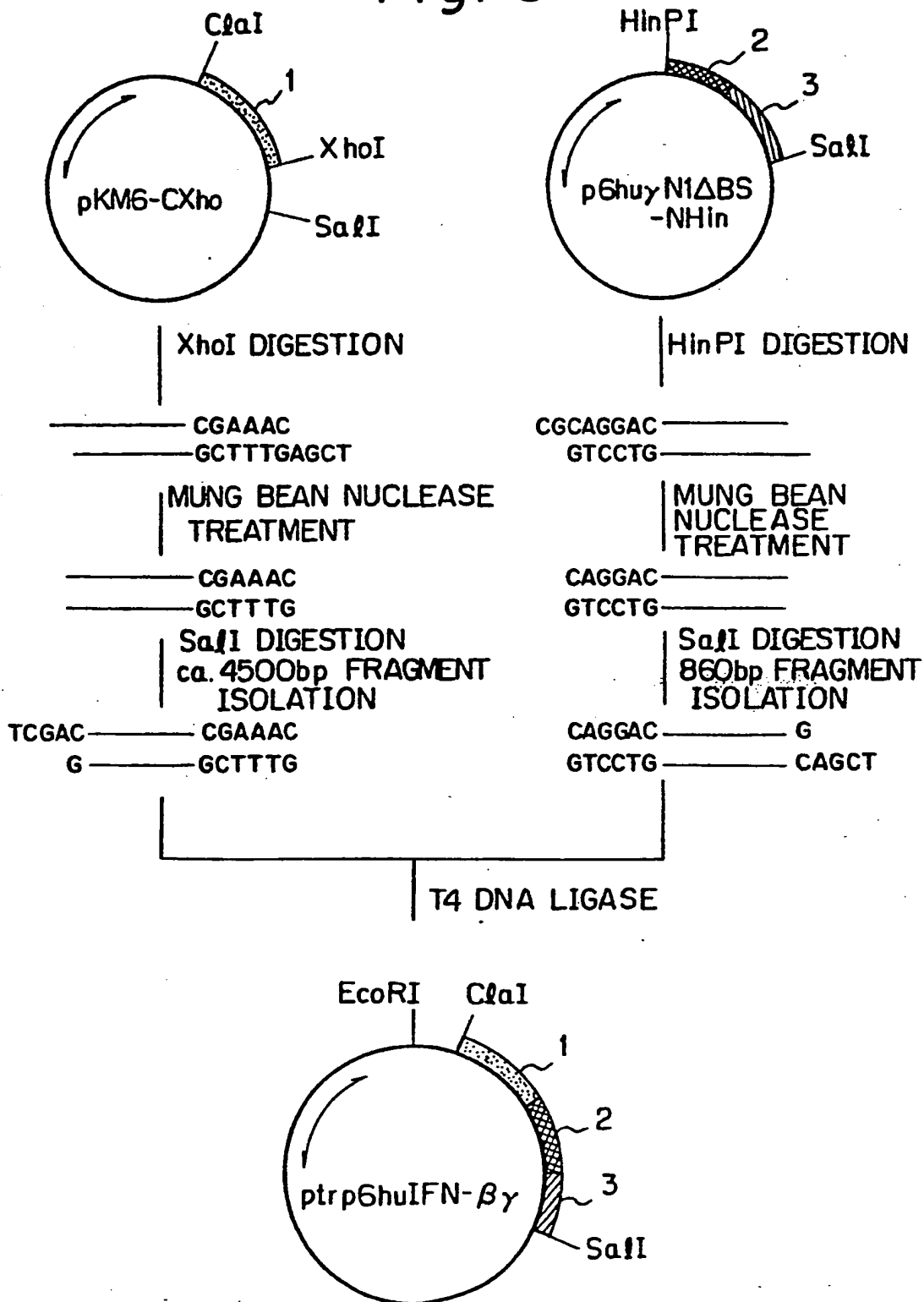
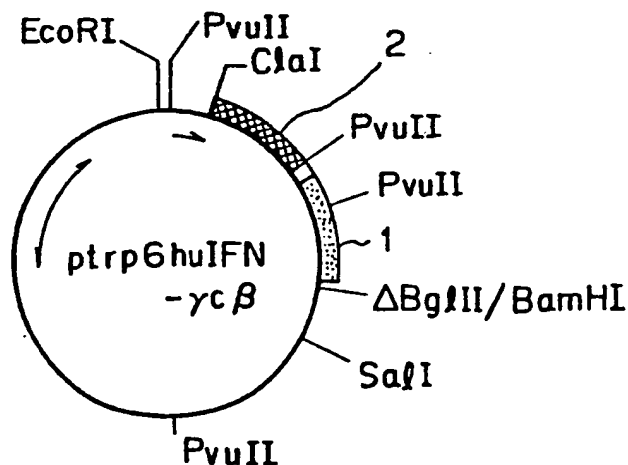
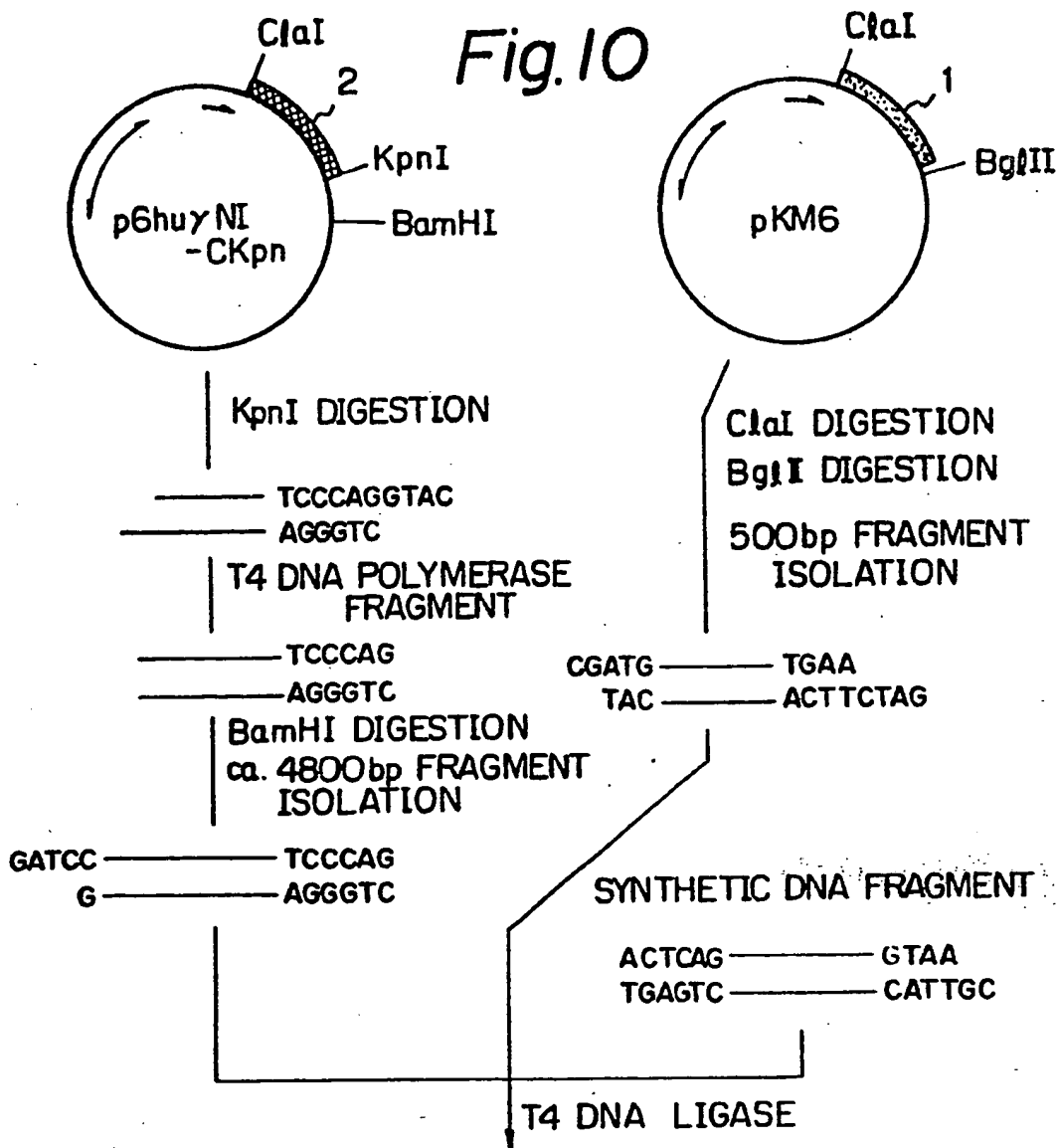




Fig. 10



*Fig. 11*

T Q L G Q P K A A K S V T

ACTCAGCTGGGCCAGCCGAAAGCTGCTAAGTCGGTAA

TGAGTCGACCCGGTCGGCTTCGAGCATTCAGCCATTGC

PvuII

Fig. 12

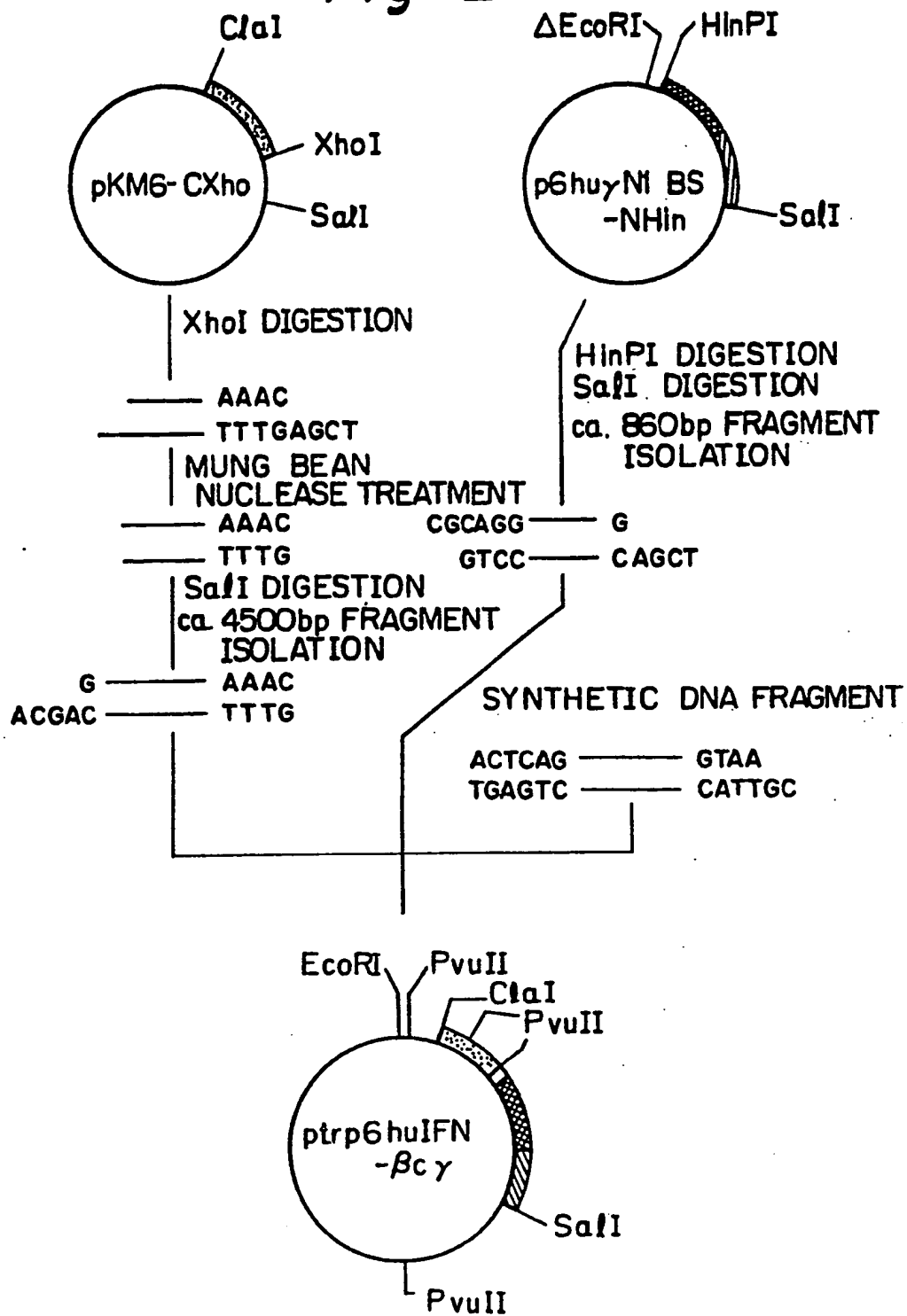


Fig. 13

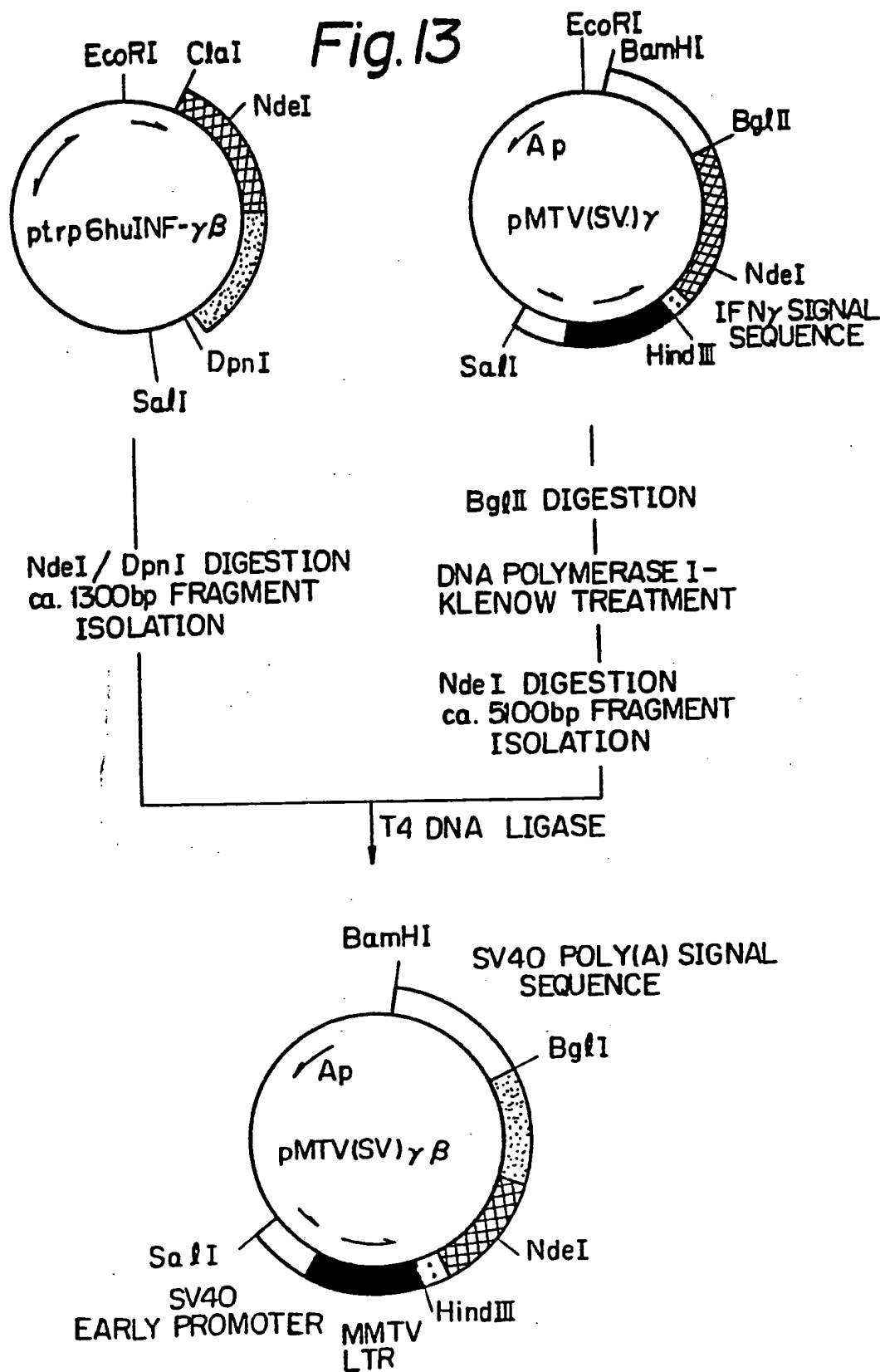


Fig. 14

